Hormonal Activity of UV Filters in Aquatic Ecosystems

Dissertation

zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Petra Y. Kunz

von

Maur ZH

Promotionskomitee

Prof. Dr. Jakob Pernthaler (Vorsitz)

Prof. Dr. Beat Keller

PD Dr. Karl Fent (Leitung der Dissertation)

Zürich, 2006

For

ULK and LMK

CONTENTS

SUMMARY

UV filters are widely and increasingly used in sunscreens for direct protection of the skin, in a variety of cosmetic products and other materials. Organic UV filters have environmental relevance, because of their demonstrated occurrence in aquatic ecosystems and their estrogenicity, antiestrogenic and antiandrogenic activity in mammalian systems. However, it remains unclear whether environmental concentrations of UV filters negatively affect the hormone system of aquatic organisms, and the ecotoxicological risk is unknown.

This dissertation was aimed at filling some of these significant gaps by investigating the hormonal activity of UV filters towards aquatic organisms by the use of a series of ecotoxicological *in vitro* and *in vivo* assays. The overall goal was to evaluate the estrogenic, aniestrogenic, androgenic and antiandrogenic activity *in vitro* of a series of single UV filters and estrogenic mixtures activities thereof. In addition the estrogenic and thyroid activity *in vivo* was evaluated in order to understand the hormonal activity and ecokinetics in important aquatic organisms, such as fish and frogs.

All 19 compounds analysed *in vitro* showed hormonal activities, surprisingly most of them multiple activities. Ten UV filters possessed estrogenic, 14 antiestrogenic, 6 androgenic and 17 antiandrogenic activities in recombinant yeast. Similar estrogenic activities were found in the two yeast systems, carrying either a hERα but also toward rtERα. To our surprise most UV filter mixtures are synergistic, especially when mixed at their NOEC.

Our experiments *in vivo* in juvenile fathead minnows showed that three UV filters were estrogenic *in vivo* and that the *in vitro* systems have a predictive value. The strongest of these UV filters, 3BC, adversely affected reproduction in fish. On the other hand the UV filters 4MBC and 3BC did not negatively affect metamorphosis and sex development of frogs at concentrations expected to be found in the environment.

Our study reveals a novel and more detailed picture of the hormonal activity of UV filters, as single compounds and in mixtures, *in vitro* and *in vivo.* It discloses unexpected multiple hormonal activities and synergistic properties of these compounds *in vitro* and an unexpected negative effect on fish reproduction by the UV filter 3BC at nearly environmentally relevant concentrations *in vivo*. Hence, diverse hormonal activities of UV filters found in this study are of significant scientific and practical interest.

ZUSAMMENFASSUNG

UV-Filter finden zum Schutze der Haut eine breite und zunehmende Anwendung in Sonnenschutzmitteln, unterschiedlichsten kosmetischen Produkten und anderen Materialien.
Organische UV-Filter gewannen an Umweltrelevanz wegen nachgewiesener Rückstände im aquatischen Ökosystem und ihrer östrogenen, antiöstrogenen, und antiandrogenen Aktivitäten in Säuger-Systemen. Trotzdem ist es bisher weitgehend unklar ob sich diese Umweltkonzentrationen negativ auf das Hormonsystem aquatischer Organismen auswirken und auch das ökotoxikologische Risiko das von diesen Substanzen ausgeht ist unbekannt.
Das Ziel dieser Dissertation war es, einige dieser relevanten Lücken zu füllen indem eine Serie von ökotoxikologische *In vitro*- und *In vivo*-Untersuchungen durchgeführt wurden. *In vitro* wurde die östrogene, antiöstrogene, androgene and antiandrogene Aktivität von einer Serie von UV-Filtern, sowie auch Mischungskombinationen davon, untersucht. Die östrogene und thyroide Aktivitäten dieser Substanzen wurde ausserdem *in vivo* untersucht um die hormonelle Aktivität und Ökokinetik in relevanten aquatischen Organismen, wie Fischen und Fröschen, zu verstehen.
Alle 19 untersuchten Substanzen besassen *in vitro* hormonelle Aktivitäten, überraschenderweise die meisten davon multiple. Zehn UV-Filter zeigten in der rekombinanten Hefe östrogene Aktivität, 14 antiöstrogene, 6 androgene und 17 antiandrogene Aktivität. Ähnliche östrogene Aktivitäten könnten in den beiden Hefesystemen nachgewiesen werden, ob sie nun einen a hERα oder einen rtERα trugen. Zu unserer Überraschung zeigtne die meisten UV-Filter Mischungen synergistische Aktivität, vor allem wenn sie bei NOEC Konzentrationen gemischt wurden.
Unsere in vivo Experimente mit juvenilen Dickkopfelritzen zeigten, dass 3 UV-Filter auch *in vivo* östrogene Aktivität besitzen und dass die benutzten *In vitro*-Systeme prädikativen Wert haben. Der stärkste dieser UV-Filter, 3BC, beeinträchtigte negativ das Fortpflanzungsverhalten in Fischen. Andererseits hatten die UV-Filter 4MBC und 3BC, in Konzentrationen die in der Umwelt zu erwarte wären, keine Auswirkungen auf die Metamorphose und Geschlechtsentwicklung beim afrikanischen Krallenfrosch
Die Resultate unserer Studie zeichnen ein neues, detaillierteres Bild der hormonellen Aktivität von UV-Filtern, bezüglich Einzelsubstanzen wie auch Mischungen, *in vitro* wie *in vivo*. *In vitro* sie zeigt die unerwarteten multiplen hormonellen Aktivitäten dieser Substanzen wie auch deren synergistische Eigenschaften in Mischungen. *In vivo* der UV-Filter 3BC führte zu unerwarteten negativen Fortpflanzungs-Effekten bei Fischen bei schon beinahe umweltrelevanten Konzentrationen. Die diversen hormonellen Aktivitäten von UV-Filtersubstanzen, die in dieser Arbeit aufgedeckt wurden, zeigen, dass diese Substanzen von wissenschaftlichem wie auch praktischem Interesse sind.

Chapter 1

General Introduction

Short introduction on endocrine disrupting chemicals

Chemicals present in the environment that possess the ability to disrupt the endocrine system of humans and animals, are of high ecotoxicological importance. Potentially the can affect the survival of populations and even the species. The identification of endocrine disrupting chemicals (EDC) has increased our knowledge about potential hazards and effects on reproduction as well as their mechanism of action. Organisms exposed to EDC can suffer from several effects. The development and quality of sperms and eggs can be negatively affected, as well as sex development and determination. Reproductive behaviour can be altered, fertility reduced and the biological sex reversed. Up to now at least 150 chemicals were identified, which interact with the endocrine system and may negatively affect reproduction (Damstra et al., 2002).
Thus far, endocrine disruption has been mostly concerned with estrogenic compounds because of the discovery that certain chemicals and sewage effluents were estrogenic and field studies showed that estrogenic effects occur in wild fish (Tyler et al., 1998). Only in the last few years, antiandrogenic compounds (Kelce et al., 1995; Vinggaard et al., 2005), and androgenic activities in the aquatic environment (Thomas et al., 2002) were identified, originating for example from kraft mill effluents . In fish polycyclic musks have found to be antiestrogenic (Schreurs et al., 2004), but better known are antiandrogenic activities of compounds like p,p'-DDE, vinclozolin and fenarimol (Vinggaard et al., 2005). Relatively few environmental contaminants have been shown to exhibit antagonistic besides agonist activity to steroid hormone receptors. For instance, alkylphenols elicited estrogenic and antiandrogenic activity *in vitro* (Sohoni and Sumpter, 1998), similarly to bisphenol A (Lee et al., 2003) and methoxychlor , to name a few chemicals. In the fields of endocrinology and pharmacology, on the other hand, multiple hormonal activities were investigated extensively, especially on the receptor level. For instance drugs targeted to the estrogen receptor (ER) were found to be selective receptor modulators (SRM's, for a detailed review see (Smith and O'Malley, 2004)) with partial agonistic activities towards the receptor, like the weakly estrogenic tamoxifen which acts antiestrogenic in the presence of estradiol (E2) (Jordan et al., 1987). Few studies have investigated multiple endocrine disrupting mechanisms of environmental contaminants. The pyrethroid insecticide fenvalerate was found to possess estrogenic as well as anti-progesteronic activity *in vitro* (Garey and Wolff, 1998). Pesticides have been shown to have agonist and/or antagonists activities with one or more receptors (Andersen et al., 2002; Kojima et al., 2004). Despite this knowledge, most chemicals found in the environment have not been analysed systematically either for agonist and antagonist activities, or different steroid receptors. Therefore, it remains elusive to what extent multiple hormonal activities are a common feature of hormonally active compounds.

UV filters – Application, use and environmental concentrations

This dissertation focuses on organic ultraviolet absorbing chemicals (UV filters), a group of chemicals which is currently of concern because of the demonstrated hormonal activity. UV filters are widely used in sunscreens for direct protection of the skin against erythema and cancer, in a variety of cosmetic products and in the UV protection of plastic products. They are added to sunscreens and cosmetics including lipsticks, skin lotions, hair sprays, hair dyes, shampoos, and numerous other products like moldings, jet ink, tyres and fabrics contain increasing amounts of UV filters protecting from ultraviolet (UV) radiation. Either organic UV filters, or inorganic micropigments (ZnO, TiO2), or combination of both are applied in order to absorb, scatter and reflect UV light. Increased sunlight protection factors are being used for preventing negative effects on the human skin, which generally requires higher percentages of UV filters in the products.

Commonly, combinations of several different UV filters are added to sunscreens, cosmetics and other materials, depending on the desired protection factor and range. By using sunscreens and personal care products different UV filters are applied on human skin, thus it is no surprise that humans can be exposed to UV filters via dermal absorption or food. Residues of benzophenone-3 (BP3) and octyl methoxycinnamate (OMC) were found in human breast milk samples up to 445 ng/g lipid (Hany and Nagel, 1995). In human urine, BP3 and its metabolite benzophenone-1 (BP1) have been detected 4 h after application of commercially available sunscreen products to the skin (Felix et al., 1998).

However, the high consumption volumes combined with critical physico-chemical properties such as environmental persistence and high lipophilicity (log Kow 3-7), make UV filters also of potential environmental concern. Consequently it is not surprising that residues of UV filters have been detected in the environment, particularly in the aquatic environment. They enter the aquatic system either directly into surface water via recreational activities (bathing) or indirectly via wastewater. Hence residues of several UV filters have been detected in lake and river water in the range of 2-80 ng/L (Poiger et al., 2004) and in treated wastewater up to 2300 ng/L (Balmer et al., 2005). In southern California two UV filters (benzophenone, octyl methoxycinnamate (OMC)) were found in the range of 0.26 to 5.61 μg/L in raw and treated drinking water (Loraine and Pettigrove, 2006) .

As UV filters are most likely relatively stable in the aquatic environment (Poiger et al., 2004; Balmer et al., 2005) they are critical for bioaccumulation. Indeed, residues of several UV filters have been detected at concentrations of 21-3100 ng/g lipid in fish (Balmer et al., 2005). In lake fish four different UV filters were identified in a recent monitoring study In recent monitoring studies in lake fish 4-methylbenzylidene camphor (4MBC), benzophenone-3 (BP3), OMC, and octocrylen (OC) were found up to concentrations of 2’400 ng/g (Balmer et al., 2005; Buser et al., 2006).

UV filters as endocrine disrupting chemicals

Besides being present in the environment, UV filters recently gained importance because of their reported estrogenic activity *in vitro* and *in vivo.* Some UV filters were found to be estrogenic *in vitro* in MCF-7 cells , recombinant cell lines (Schreurs et al., 2002; Mueller et al., 2003) and recombinant yeast systems carrying the human ERα (Routledge and Sumpter, 1997; Schultz et al., 2000; Kunz and Fent, submitted). Estrogenic activity has also been observed *in vivo* in rats (Durrer *et al.* 2005; Schlumpf *et al.* 2001; Seidlová-Wuttke *et al.* 2004) and fish. In rats 3BC, 4MBC, OMC, benzophenones-2 (BP2), bezophenone-3 (BP3) and 4,4-dihydroxybenzophenone (4DHB) elicited estrogenic effects (Schlecht et al., 2004). In rainbow trout 3BC led to vitellogenin induction (Holbech et al., 2002), whereas 4MBC and OMC were found to be estrogenic in male medaka (Inui et al., 2003). Besides being estrogenic, some UV filters such 4MBC, homosalate (HMS) and BP3 showed antiestrogenic activity (Schreurs et al., 2002; Mueller et al., 2003), and BP3 and HMS had antiandrogenic activity *in vitro* (Ma et al., 2003; Schlumpf et al., 2004). However, it is not known whether multiple hormonal activities are a common feature of these compounds.

Despite the increasing amount of literature on environmental residues and endocrine disruption properties of UV filters it remains unclear whether environmental concentrations of UV filters negatively affect the hormone system of aquatic organisms. Due to the lack of data on the ecotoxicological and hormonal activity of UV filters, currently an ecotoxicological risk assessment for aquatic organisms is unthinkable unless sufficient data are generated in the near future.

Objectives

This dissertation is aimed at filling these significant gaps by the use of a series of ecotoxicological *in vitro* and *in vivo* assays. The overall goal is to evaluate the estrogenic, antiestrogenic, androgenic and antiandrogenic activity of a series of single UV filters. Furthermore estrogenic mixtures activities *in vitro* will be evaluated. In addition, the estrogenic and thyroid activity *in vivo* will be assessed in order to understand the hormonal activity and ecokinetics in important aquatic organisms such as fish and frog.

In order to shed some light on other possible endocrine properties of UV filters, especially as these compounds are found in surface waters and fish (Balmer et al., 2005; Buser et al., 2006), we screened 19 compounds for their hormonal activity *in vitro*. We used a reporter gene assay with recombinant yeast carrying either a human estrogen (hERα) or androgen (hAR) receptor in order to investigate the estrogenic, antiestrogenic, androgenic and antiandrogenic activity of these UV filters. We compared the estrogenic activities of UV filters found in the hERα with

their estrogenic activities in a recombinant yeast system carrying a rainbow trout ER (rtERα). We also evaluated their predictive value when compared with results of *in vivo* experiments in fish.

With the UV filters found to be estrogenic towards the hERα we furthermore conducted mixture experiments where UV filters were combined at low and no observed effect concentrations in order to find out whether and how the estrogenic and/or antiestrogenic activity of single UV filters contribute to mixture effects.

We further examined the estrogenic activity of 9 major UV filters in fish *in vivo*, which are important representatives of aquatic organisms, and evaluated whether the estrogenic activity *in vitro* translates to *in vivo*. We first performed a short-term experiment with juvenile fathead minnows (*Pimephale promelas*), in order to investigate whether these nine UV filters lead to vitellogenin (VTG) induction as a biomarker for estrogenic activity. Second we investigated whether the most potent UV filter of the short-term experiment, 3BC, adversely affects reproduction in fathead minnows.
In addition we investigated in frogs, *Xenopus laevis*, as another important representative for aquatic organisms, whether two UV filters, 4MBC and 3BC, interfere with the thyroid and sex hormone system during frog metamorphosis.

In order to estimate actual effect concentrations in ecotoxicological experiments with fish and frog we modified an existing GC/MS method, (Poiger et al., 2004; Balmer et al., 2005) for the analysis of the UV filters 4MBC and 3BC. We subsequently extended it for the analysis of 9 different UV filters used in the fish experiments by developing a method based on solid phase extraction followed by analysis with HPLC-DAD.

Ultimately the aim of the thesis was to gain a better knowledge on the hormonal activities of UV filters by using *in vitro* and *in vivo* systems. These data should help to better understand the potential hazards and risks associated with these compounds in aquatic ecosystems.

The thesis is presented in 8 chapters, examining hormonal activity of UV filters toward the aquatic ecosystem as follows:

CHAPTER 1 gives a general introduction to the topic and the study

CHAPTER 2 addresses the hormonal activity of 19 commonly used UV filters *in vitro* by employing a recombinant yeast system carrying either a human estrogen (hERα) or androgen (hAR) receptor. In a systematic manner the UV filters are screened for estrogenic, antiestrogenic, androgenic and antiandrogenic activity. Furthermore one estrogenic UV filter,

ethyl 4-aminobenzoate (Et-PABA), was evaluated for its estrogenicity in a recombinant yeast assay carrying a rainbow trout ER (rtERα) and for vitellogenin induction *in vivo* in juvenile fish (*Pimephales promelas*). This approach enables to evaluate whether results in the hERα are transferable to the rtERα *in vitro*, and to what extend this may translate to activity in fish *in vivo*.

CHAPTER 3 addresses the hormonal activity of UV filter mixtures. For the mixture components commonly used UV filters were selected, which were demonstrated to be pure hERα agonists (BP1, BP2, 4DHB and Et-PABA) or partial hERα agonists (BP3, BS, PS and 3BC) *in vitro* in Chapter 1. The mixture activities of 2, 4 and 8 UV filters, with and without E2, are investigated at different effect levels and at the no observed effect level concentration (NOEC). This question is important as commonly combinations of several different UV filters are added to sunscreens and cosmetics and are applied on human skin. As they enter the aquatic ecosystem as mixtures; increasing attention should be paid on the mixture effects.

CHAPTER 4 addresses the modification of an existing GC/MS method (Poiger et al., 2004; Balmer et al., 2005) and its extension for the analysis of 9 different UV filters in experimental waters by a method based on solid phase extraction followed by analysis with HPLC-DAD. Accurate detection of UV filters in water of ecotoxicological experiments with fish and frogs is crucial for the determination of exposure and effect concentrations.

CHAPTER 5 addresses the estrogenic activity of commonly used UV filters in fish and elucidates whether estrogenic activity of chemicals is best assessed by the use of a tiered approach using a combination of *in vitro* and *in vivo* assays of the same phyla. Therefore estrogenicity of 8 UV filters is assessed in juvenile fathead minnows (*Pimephales promelas*), by means of vitellogenin induction. The estrogenic activity in fish is then compare with *in vitro* activities of these UV filters in two *in vitro* systems carrying either a fish or the human ERα.

CHAPTER 6 evaluates whether a common UV filter, 3BC, adversely affects reproduction in fathead minnows. In this thesis, 3BC was previously found to be estrogenic *in vitro* and *in vivo* (Chapter 1 and 4). Adult fathead minnows were exposed for 21 days to five increasing concentrations of 3BC and parameters for endocrine disruption, such as fecundity, vitellogenin induction, secondary sex characteristics and gonad histology were investigated.

CHAPTER 7 elucidates possible activities of the two UV screens, 4-methylbenzylidene camphor (4MBC) and 3-benzylidene camphor (3BC), towards the thyroid and sex hormone system of frogs *Xenopus laevis*. It investigates whether these UV filters negatively affect metamorphosis, which is regulated by the thyroid system, and sex development of frogs at concentrations expected to be found in the environment.

CHAPTER 8 gives a general conclusion and outlook

Our findings reveal a novel picture of the hormonal activities of UV filters and address the question of their modes of action. All 19 compounds analysed *in vitro* showed hormonal activities, surprisingly most of them multiple activities. Ten UV filters possessed estrogenic, 14 antiestrogenic, 6 androgenic and 17 antiandrogenic activities in recombinant yeast. Similar estrogenic activities were found in the two yeast systems, carrying either a hERα but also toward rtERα. To our surprise most UV filter mixtures are synergistic, especially when mixed at their NOEC.

Our experiments *in vivo* in juvenile fathead minnows showed that *in vitro* systems had a predictive value. It did not predict false negatives and three UV filters were estrogenic *in vivo* leading to significant vitellogenin induction. The strongest of these UV filters, 3BC, adversely affected reproduction in fish in a dose-dependent manner. On the other hand the UV filters 4MBC and 3BC did not negatively affect metamorphosis and sex development of frogs at concentrations expected to be found in the environment.

Our *in vitro* and *in vivo* findings on hormonal activity of UV filters indicate the need for a tiered approach combining *in vitro* and *in vivo* assessments for ecological risk assessment. They raise the question whether UV filter residues found in the environment are potentially able to adversely affect aquatic organisms with their multiple hormonal activities. In particular the substantial mixture effects even though each UV filter was present below its NOEC level, and the ability of 3BC to negatively affect fish reproduction at rather low concentrations demonstrate potential ecotoxicological hazard and risks and the environmental importance of these compounds.

References

Andersen, H. R., Vinggaard, A. M., Rasmussen, T. H., Gjermandsen, I. M., Bonefeld-Jorgensen, E. C., 2002. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity *in vito*. Toxicol. Appl. Pharmacol. 179, 1-12.

Balmer, M., Buser, H. R., Müller, M. D., Poiger, T., 2005. Occurrence of some organic UV filters in wastewater, in surface waters, and in fish from Swiss lakes. Environ. Sci. Technol. 39, 953-962.

Buser, H. R., Balmer, M. E., Schmid, P., Kohler, M., 2006. Occurence of UV filters 4-methylbenzylidene camphor and octocrylene in fish from various swiss rivers with inputs from wastewater treatment plants. Environ. Sci. Technol. 40, 1427-1431.

Damstra, T., Barlow, S., Bergmann, A., Kavlock, R., Van Der Kraak, G., 2002. Global assessment of the state-of-the science of endocrine disruptors. Geneva, Word Health Organisation, International Health Programme on Chemical Safety.

Felix, T., Hall, B. J., Brodbelt, J. S., 1998. Determination of benzophenone-3 and metabolites in water and human urine by solid-phase microextraction and quadrupole ion trap GC-MS. Anal. Chim. Acta 371, 195-203.

Garey, J., Wolff, M. S., 1998. Estrogenic and antiprogestagenic activities of pyrethroid insecticides. Biochem. Biophys. Res. Com. 251, 855-859.

Hany, J., Nagel, R., 1995. Nachweis von UV-Filtersubstanzen in Muttermilch. Deut. Lebensm.-Rundsch. 91(11), 341-345.

Holbech, H., Norum, U., Korsgaard, B., Bjerregaard, P., 2002. The chemical UV-filter 3-benzylidene camphor causes an oestrogenic effect in an *in vivo* fish assay. Pharmacol. Toxicol. 91, 204-208.

Inui, M., Adachi, T., Takenaka, S., Inui, H., Nakazawa, M., Ueda, M., Watanabe, H., Mori, C., Iguchi, T., Miyatake, K., 2003. Effect of UV-screens and preservatives on vitellogenin and choriogenin production in male medaka (*Oryzias latipes*). Toxicology 194, 43-50.

Jordan, V. C., Phelps, E., Lindergreen, J. U., 1987. Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res. Treat. 10, 31-35.

Kelce, W. R., Stone, C. R., Laws, S. C., Gray, L. E., Kemppainen, J. A., Wilson, E. M., 1995. Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. Nature 375, 581-585.

Kojima, H., Katsura, E., Takeuchi, S., Niiyama, K., Kobayashi, K., 2004. Screening for estrogen and androgen receptor activities in 200 pesticides by *in vitro* reporter gene assays using Chinese Hamster ovary cells. Environ. Health Perspect. 112(5), 524-531.

Kunz, P. Y., Fent, K., submitted. Multiple hormonal activities of UV filters in vitro.

Lee, H. J., Chattopadhyay, S., Gong, E. Y., Ahn, R. S., Lee, K., 2003. Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. Toxicol. Sci. 75, 40-46.

Loraine, G. A., Pettigrove, M. E., 2006. Seasonal variations in concentrations of pharmaceuticals and personal care products in drinking water and reclaimed wastewater in southern california. Environ. Sci. Technol. 40, 687-695.

Ma, R., Cotton, B., Lichtensteiger, W., Schlumpf, M., 2003. UV filters with antagonistic action at androgen receptors in the MDA-kb2 cell transcriptional-activation assay. Toxicol. Sci. 74, 43-50.

Mueller, S. O., Kling, M., Firzani, P. A., Mecky, A., Duranti, E., Shields-Botella, J., Delansorne, R., Borschard, T., Kramer, P. J., 2003. Activation of estrogen receptor α and ERβ by 4-methylbenzylidene-camphor in human an rat cells: comparison with phyto- and xenoestrogens. Toxicol. Lett. 142, 89-101.

Poiger, T., Buser, H. R., Balmer, M., Bergqvist, P. A., Müller, M. D., 2004. Occurrence of UV filter compounds from sunscreens in surface waters: regional mass balance in two Swiss lakes. Chemosphere 55, 951-963.

Routledge, E. J., Sumpter, J. P., 1997. Structural features of alkylphenolic chemicals associated with estrogenic activity. J. Biol. Chem. 272(6), 3280-3288.

Schlecht, C., Klammer, H., Jarry, H., Wuttke, W., 2004. Effects of estradiol, benzophenone-2 and benzophenone-3 on the expression pattern of the estrogen receptors (ER) alpha and beta, the estrogen receptor-related receptor 1 (ERR1) and the aryl hydrocarbon receptor (AhR) in adult ovariectomized rats. Toxicology 205, 123-130.

Schlumpf, M., Schmid, P., Durrer, S., Conscience, M., Maerkel, K., Henseler, M., Gruetter, M., Herzog, I., Reolon, S., Ceccatelli, R., Faass, O., Stutz, E., Jarry, H., Wuttke, W., Lichtensteiger, W., 2004. Endocrine acitivity and developmental toxicity of cosmetic UV filters - an update. Toxicology 205, 113-122.

Schreurs, R. H., Lanser, P., Seinen, W., Van der Burg, B., 2002. Estrogenic activity of UV filters determined by an in vitro reporter gene assay and in vivo transgenic zebrafish assay. Arch. Toxicol. 76, 257-261.

Schreurs, R. H., Legler, J., Artola-Garicano, E., Sinnige, T. L., Lanser, P. H., Seinen, W., Van der Burg, B., 2004. In vitro and in vivo antiestrogenic effects of polycyclic musks in zebrafish. Environ. Sci. Technol. 38(4), 997-1002.

Schultz, T. W., Seward, J. R., Sinks, G. D., 2000. Estrogenicity of benzophenones evaluated with a recombinant yeast assay: Comparison of experimental and rules-based predicted activity. Environ. Toxicol. Chem. 19, 301-304.

Smith, C. L., O'Malley, B. W., 2004. Coregulator function: A key to understanding tissue specificity of selective receptor modulators. Endocr. Rev. 25(1), 45-71.

Sohoni, P., Sumpter, J. P., 1998. Several environmental oestrogens are also anti-androgens. J. Endocrinol. 158(3), 327-339.

Thomas, K. V., Hurst, M. D., Matthiessen, P., McHugh, M., Smith, A., Waldock, M. J., 2002. An assessment of in vitro androgenic activity and the identification of the environmental androgens in the United Kingdom estuaries. Environ. Toxicol. Chem. 21, 1456-1461.

Tyler, C. R., Jobling, S., Sumpter, J. P., 1998. Endocrine disruption in wildlife: a critical review of the evidence. Crit. Rev. Toxicol. 28(4), 319-61.

Vinggaard, A. M., Jacobsoen, H., Metzdorff, S. B., Andersen, H. R., Nellemann, C., 2005. Antiandrogenic effects in short-term in vivo studies of the fungicide fenarimol. Toxicology 207(1), 21-34.

Acknowledgments

First of all, I want to express my gratitude to my supervisor Karl Fent who gave me the opportunity to do this thesis. He introduced me to the fascinating world of ecotoxicology and guided me through a very interesting topic. His trust in me, his flexibility and open mindedness made the last four years to a time in my life, that I enjoyed very much. His moral and practical support helped me in times, when things did not go so smoothly, and also in times when I was too optimistic. I appreciated our inspiring scientific discussions about the study-design, amendments, future perspectives, and how to write a good discussion, and I also appreciated our non-scientific discussions very much. Especially, I highly appreciate the time and effort he put into critically reading and reviewing of all my writings, in particularly at the end of my thesis, so that I was able to finish "in time". I could not have wished for a better supervisor.

I also want to express my gratitude to Prof. Friedrich Jüttner and Prof. Jakob Pernthaler, who gave me the feeling to belong to the Limnological Station, although I did my thesis in Basel. I highly appreciated their interest in my thesis and their repeated invitations to give a talk and keep them up to date. Jakob Pernthaler I furthmore would lik to thank for his efforts at the end of my thesis. I appreciate that very much!

I am also very grateful to the Insitute of Environmental Technology and the Department of Chemistiy and for giving me lab and work facilities and supported me. Especially I am very grateful to Markus Wolf, Thomas Heim, Ernst Hungerbühler, Hansrudolf Schmutz and Daniel Gygax who always gave me the feeling, that I was one of them.

A great thank you goes to Hanruedi Schmutz and his analytics group for introducing me to SPE, HPLC and GC/MS and for valuable and great support in developing and testing several analytical methods in order to analyse my water and fish samples. Especially I would like to thank Beny Gygax, who helped me a lot with his critical and constructive suggestions and his dry humor.

Another great thank you goes to the people of bioanalytics lab for supporting me in every way. Especially Peter Spies, without whom I would be still unaware of how to use a pipette and who was the best room mate one could wish for!

I would also like to thank the ecotoxicology group members Karl, Daniel, Anna, Christin, Claudia and Armin for your support and encouragement when needed. Anna, Daniel, Christin and Claudia, you are probably the best "fish test determination group" in the world. Daniel, thanks for making very serious jokes and being "Bündner", Anna thanks for being also a little paranoid, Christine thanks for being my best "fish test partner" and "never tired egg counter", and Claudia thanks for helping in fish matters and making me "sporty".

I would also like to sincerely thank Héctor Galicia and the Springborn Smithers Laboratories for providing an excellent laboratory to conduct all the *in vivo* experiments. Especially I would like to thank Thomas Gries for his support and advice during my "fieldwork" at Springborn. Furthermore my thanks go also to Tony, Ceo, Lea, Karin, Alessandra, Daniela and all other Springborn people, who helped whenever I got lost and always had time to answer one of my questions, and for giving my some social life in Horn.

And more thanks go to all the other chemistry and IfU people who contributed to this thesis by just being as nice as they are: Illu, René, Eric, Viviane, Roger, Antje and Astha.

Furthermore I want to say thank you to all people who contributed to this thesis in their own wonderful way by being part of my life. Especially Rosine queen of frog dissections and other things, Vre, Sabine, Gabrie, Chandra, Nîck, Claudi, and all the others, whose names are too numerous to be listed here, they are and will be remembered. Deep thanks go to my parents and my brother, who let me grow and supported me.

And a very special thank goes to Lukas, who is the true home of my life.

Chapter 2

Activity of UV filters towards the estrogen and androgen receptor and comparison of *in vitro* and *in vivo* activity of ethyl 4-aminobenzoate in fish

Petra Y. Kunz and Karl Fent

accepted in *Aquatic Toxicology*

Abstract

UV filters have been detected in surface water, wastewater and fish, and some of them are estrogenic in fish. At present, little is known about their additional hormonal activities in different hormonal receptor systems despite their increasing use and environmental persistence. Besides estrogenic activity, UV filters may have additional activities, both agonistic and antagonistic in aquatic organisms. In our study, we investigate a series of UV filters for multiple hormonal activities *in vitro* in human receptor systems and evaluate the predictive value of these findings for the activity in fish *in vitro* and *in vivo*. First we systematically analysed the estrogenic, antiestrogenic, androgenic, and antiandrogenic activity of 18 UV filters and one metabolite *in vitro* at non-cytotoxic concentrations with recombinant yeast systems carrying either a human estrogen (hERα or androgen receptor (hAR). All 19 compounds elicited hormonal activities, surprisingly most of them multiple activities. We found 10 UV-filters having agonistic effects towards the hERα. Surprisingly, we identified for the first time 6 UV filters with androgenic activities and many of them having pronounced antiestrogenic and antiandrogenic activities. As much as 17 compounds inhibited 4,5-dihydrotestosterone activity in the hAR assay, while 14 compounds inhibited estradiol activity in the hERα assay, indicating antiandrogenic and antiestrogenic activity, respectively. In particular, the antiandrogenic activities of phenyl- and benzyl salicylate, benzophenone-1 and -2, and of 4-hydroxybenzophenone were higher than that of flutamide, a known hAR antagonist.

In a second series of experiments, we investigated the predictive power of the hERα assay for aquatic organisms by further investigating the estrogenic UV filter ethyl 4-aminobenzoate (Et-PABA) *in vitro* and *in vivo* in fish. Et-PABA showed estrogenic activity in a recombinant yeast system carrying the rainbow trout estrogen receptor (rtERα with higher activity than in the hERα assay. In addition, Et-PABA induced vitellogenin after 14 d of exposure in juvenile fathead minnows at 4’394 µg/L.Our study shows estrogenic activity of this UV filter in fish both *in vitro* and *in vivo*. In conjunction with *in vitro* human receptor-based systems our results give a more detailed picture about distinct hormonal activities of UV filters occurring in aquatic systems. We conclude that receptor-based assays are important for *in vitro* assessment of UV-filters prior to or concurrently with *in vivo* assays, which ultimately provide data for the environmental risk assessment of these important personal care products.

Introduction

Thus far, endocrine disruption has been mostly concerned with estrogenic compounds because of the discovery that certain chemicals (Soto et al., 1991) and sewage effluents were estrogenic and field studies showed that estrogenic effects occur in wild fish (Tyler et al., 1998). Only in the last few years, antiandrogenic compounds (Kelce et al., 1995; Vinggaard et al., 2005), and androgenic activities in the aquatic environment (Thomas et al., 2002) were identified, originating for example from kraft mill effluents (Parks et al., 2001). In fish polycyclic musks have found to be antiestrogenic (Schreurs et al., 2004), but better known are antiandrogenic activities of compounds like p,p'-DDE, vinclozolin (Kelce and Wilson, 1997) and fenarimol (Vinggaard et al., 2005). Thus far, relatively few environmental contaminants have been shown to exhibit antagonistic besides agonist activity at steroid hormone receptors. For instance, alkylphenols elicited estrogenic and antiandrogenic activity *in vitro* (Sohoni and Sumpter, 1998), similarly to bisphenol A (Lee et al., 2003) and methoxychlor (Maness et al., 1998), to name a few chemicals. In the fields of endocrinology and pharmacology, on the other hand, multiple hormonal activities were investigated extensively, especially on the receptor level. For instance drugs targeted to the ER were found to be selective receptor modulators (SRM's, for a detailed review see Smith and O'Malley (2004)) with partial agonistic activities towards the receptor, like the weakly estrogenic tamoxifen which acts antiestrogenic in the presence of E2 (Jordan et al., 1987). Few studies have investigated multiple endocrine disrupting mechanisms of environmental contaminants. The pyrethroid insecticide fenvalerate was found to possess estrogenic as well as anti-progesteronic activity *in vitro* (Garey and Wolff, 1998). Pesticides have been shown to have agonist and/or antagonists activities with one or more receptors (Andersen et al., 2002; Kojima et al., 2004). Despite this knowledge, most chemicals found in the environment have not been analysed systematically either for agonist and antagonist activities, or different steroid receptors. Therefore, it remains elusive to what extent multiple hormonal activities are a common feature of hormonally active compounds.

Organic ultraviolet absorbing chemicals (UV filters) are currently of concern because of the demonstrated estrogenicity of some of these compounds (Miller et al., 2001; Schlumpf et al., 2001). UV filters are widely used in sunscreens for direct protection of the skin against erythema and cancer, in a variety of cosmetic products and in the UV protection of plastic products.

Thus it is no surprise that humans can be exposed to UV filters via dermal absorption or food. Residues of BP3 and OMC were found in human breast milk samples (Hany and Nagel, 1995). In human urine, BP3 and its metabolite benzophenone-1 (BP1) have been detected 4 h after application of commercially available sunscreen products to the skin (Felix et al., 1998). But the high consumption volumes combined with critical physico-chemical properties such as

environmental persistence and high lipophilicity, make UV filters also of potential environmental concern. Recently, residues of UV filters have been found in lake and river water in the range of 2-80 ng/L (Poiger et al., 2004) and in treated wastewater up to 2300 ng/L (Balmer et al., 2005). In lake fish four different UV filters were identified in a recent monitoring study at concentrations up to 166 ng/g (lipid) 4-methylbenzylidene camphor, 123 ng/g benzophenone-3 (BP3) and 64 ng/g octyl methoxycinnamate (OMC)(Balmer et al., 2005; Buser et al., 2006).

Besides being present in the environment, UV filters recently gained importance because of their reported estrogenic activity *in vitro* (Miller et al., 2001; Schlumpf et al., 2001; Schreurs et al., 2002; Mueller et al., 2003). In juvenile fathead minnows 3BC, BP1 and BP2 led to vitellogenin induction (Kunz et al., 2006) and 3BC did as well in rainbow trout (Holbech et al., 2002), whereas 4MBC and OMC were found to be estrogenic in male medaka (Inui et al., 2003). An indication for VTG expression in rainbow trout was found after the exposure to benzophenone-3 (BP3) (Daniel Schlenk, personal communication). BP3 was also present in wastewater from New York City and the exposure of male medaka to wastewater fractions lead to VTG induction (Sapozhnikova et al., 2005). BP3 also occurred in marine sediment and exposure of male California halibut to this sediment led to VTG induction (Schlenk et al., 2005). In rats 3BC, 4MBC, OMC, BP2, BP3 and 4,4-dihydroxybenzophenone (4DHB) elicited estrogenic effects (Schlumpf et al., 2001; Yamasaki et al., 2003; Schlecht et al., 2004). Besides being estrogenic, some UV filters such 4MBC, homosalate (HMS) and BP3 showed antiestrogenic activity (Schreurs et al., 2002; Mueller et al., 2003), and BP3 and HMS had antiandrogenic activity *in vitro* (Ma et al., 2003; Schlumpf et al., 2004). However, it is not known whether multiple hormonal activities are a common feature of these compounds. In order to shed some light on other possible endocrine properties of UV filters, especially as these compounds are found in surface waters and fish (Balmer et al., 2005; Buser et al., 2006), we screened 19 compounds for additional agonistic and antagonistic activities besides estrogenicity. Therefore we employed a recombinant yeast system in order to investigate, in a systematic manner, the estrogenicity, antiestrogenicity, androgenicity and antiandrogenicity of commonly used UV filters. We used this approach, because earlier results on estrogenicity of UV filters demonstrated that the yeast assay is particularly useful for screening this group of compounds. First results indicate that it identifies possible estrogenic compounds and separates them from compounds with no estrogenic activity, averting false negative results (Kunz et al., 2006). Therefore we furthermore investigated one UV filter, ethyl 4-aminobenzoate (Et-PABA), that was found to be estrogenic in the hERα assay, for its estrogenicity in a recombinant yeast assay carrying a rainbow trout ER (rtERα). Et-PABA is also used as an anaesthetic in veterinary medicine. In addition, we analysed this compound in juvenile fathead minnows for vitellogenin induction. With this approach we were able to validate whether our findings for the hER are transferable to the rtER *in vitro*, and fish *in vivo*.

Our results reveal a novel picture of the hormonal activities of these environmentally relevant chemicals and address the question of their modes of action. All 19 compounds analysed showed hormonal activities, surprisingly most of them multiple activities. Moreover, antiestrogenicity and antiandrogenicity of UV filters are possibly even more environmentally relevant than

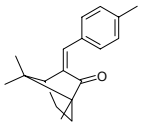
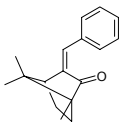
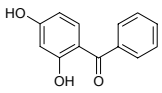
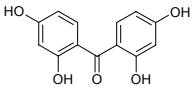
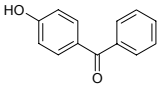
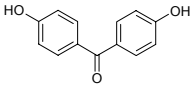
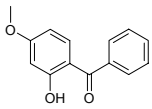
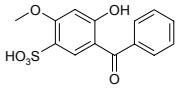
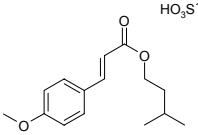
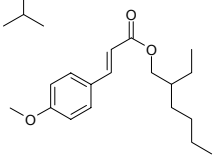
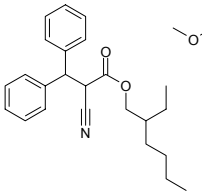
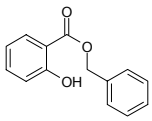
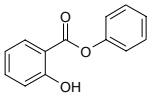
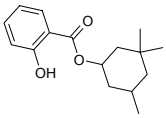
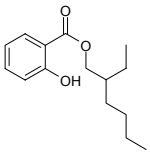
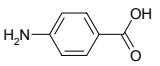
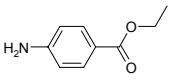
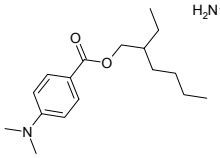
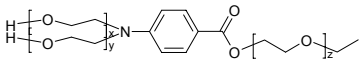
estrogenicity. Et-PABA displayed its estrogenic activity not only towards the hERα but also toward rtERα and *in vivo* in fish. These findings raise the question whether UV filter residues found in the environment are potentially able to adversely affect aquatic organisms with their multiple hormonal activities. Forthcoming studies on their effects on fish reproduction will show whether multiple hormonal activities will be of importance *in vivo*.

Material and Methods

Chemicals

17β-Estradiol (E2), 4,5-dihydrotestosterone (DHT), 4-hydroxytamoxifen (4HT) and flutamide (FT) were purchased from Fluka AG (Buchs, Switzerland). UV filters (Tab. 1) were obtained as follows. Benzophenone-1 (BP1, 2,4-dihydroxybenzophenone), benzophenone-2 (BP2, 2,2',4,4'-tetrahydroxybenzophenone), benzophenone-3 (BP3, 2-hydroxy-4-methoxybenzophenone), benzophenone-4 (BP4, 2-benzoyl-5-methoxy-1-phenol-4-sulfonic acid), 4,4'-dihydroxybenzophenone (4DHB), 4-hydroxybenzophenone (4HB), 4-aminobenzoic acid (PABA), ethyl 4-aminobenzoate (Et-PABA), benzyl salicylate (BS, benzyl 2-hydroxybenzoate), phenyl salicylate (PS, phenyl 2-hydroxybenzoate), octyl salicylate (OS, 2-ethylhexyl salicylate), octocrylene (OC, 2-cyano-3,3-diphenyl acrylic acid, 2-ethyl hexyl ester) and octyl dimethyl PABA (OD-PABA, 2-ethylhexyl 4-(dimethylamino)benzoate) were obtained from Fluka AG (Buchs, Switzerland). Octyl-methoxycinnamate (OMC, 2-ethylhexyl p-methoxycinnamate), 3-(4`-methylbenzylidene-camphor) (4MBC, 3-(4-methylbenzylidene)bornan-2-one), 3-benzylidene-camphor (3BC, 3-benzylidenebornan-2-one) and homosalate (HMS, 3,3,5-trimethylcyclohexyl salicylate) were purchased from Merck (Glattburgg, Switzerland). Ethoxylated ethyl-4-aminobenzoate (PEG25-PABA) was purchased from Induchem AG (Volketswil, Switzerland), and isopentyl-4-methoxycinnamate (IMC) was from Haarmann & Reimer (Holzminden, Germany). All purchased compounds used were >99% pure. Stock solutions were made in ethanol and stored in the dark at 4°C. Analytical grade ethanol (free of UV filter) was purchased from T.J. Baker (Stehelin AG, Basel, Switzerland). Bidestilled water was produced using a Jencons Autostill double D-ionstill (Renggli AG, Rotkreuz, Switzerland).

Table 1. Chemical structures, molecular weight and CAS numbers of compounds analysed.

Compound	Chemical structure	Molecular Weight (g/kg)	CAS
4-Methylbenzylidene camphor (4MBC)		254.37	36861-47-9
3-Benzylidene camphor (3BC)		240.34	15087-24-8
Benzophenone-1 (BP1)		214.22	131-56-6
Benzophenone-2 (BP2)		246.22	131-55-5
4-Hydroxy benzophenone (4HB)		198.22	1137-42-4
4,4'-Dihydroxybenzophenone (4DHB)		214.22	611-99-4
Benzophenone-3 (BP3)		228.25	131-57-7
Benzophenone-4 (BP4)		308.31	4065-45-6
Isopentyl-4-methoxycinnamate (IMC)		248.32	71671-10-2
Octyl-methoxycinnamate (OMC)		290.40	5466-77-3
Octocrylene (OC)		361.48	6197-30-4
Benzyl salicylate (BS)		228.25	118-58-1
Phenyl salicylate (PS)		214.22	118-55-8
Homosalate (HMS)		262.35	118-56-9
Octyl salicylate (OS)		250.33	118-60-5
Para amino-benzoic acid (PABA)		137.10	150-13-0
Ethyl-4-aminobenzoate (Et-PABA)		165.19	94-09-7
Octyl dimethyl para amino benzoate (OD-PABA)		277.41	21245-02-3
Ethoxylated ethyl 4-amino benzoate (PEG25-PABA)		165.2	113010-52-9

Abbreviations: CAS, Chemical Abstracts Service

Yeast hERα and hAR assays

Yeast estrogen assay expressing human estrogen receptor alpha (hERα assay). The estrogen-inducible expression system used is described in detail by Routledge and Sumpter (1996). In brief, the yeast (*Saccharomyces cerevisiae*) genome carries a stably integrated DNA sequence of the human estrogen receptor (hERα). Yeast cells also contain expression plasmids carrying estrogen responsive elements, regulating the expression of the reporter gene lacZ (encoding the enzyme β-galactosidase). Thus, when an active ligand (i.e. E2 or an estrogenic UV filter) binds to the receptor, β-galactosidase is synthesised and secreted into the medium, leading to a colour change of chromogenic substrate chlorophenol red ß-D-galactopyranoside (CPRG) from yellow to red.

Yeast androgen assay expressing the human androgen receptor (hAR assay). The main principles of the hAR assay are basically the same as those of the hERα assay, if not stated otherwise (for details see Sohoni and Sumpter (1998). The yeast cells contain a constitutively expressed gene for the human androgen receptor (hAR). In the presence of a ligand such as DHT or an androgenic UV filter, the androgen receptor binds to an androgen responsive element on a plasmid, thereby initiating transcription of the reporter gene lacZ and β-galactosidase activity.

Preparation of assay media. Preparation of medium compounds was done as previously described (Routledge and Sumpter, 1996). All components except $Fe_2(SO_4)_3$, inotisol, copper (II) sulphate (Fluka, Buchs, Switzerland) and CPRG (Roche, Basel, Switzerland), were purchased from Sigma (Glattbrugg, Switzerland). Ten-times concentrated stock cultures of both recombinant yeast strains were stored at -20°C in 0.5 mL aliquots. Every four months (shelf life), both yeast strains were replaced with new -20°C stock cultures. Prior to the experiments the growth medium was inoculated with 125 µL ten times concentrated yeast stock, and incubated at 28°C for 24 h on an orbital shaker. The final assay medium was prepared by seeding 50 mL fresh growth medium with $4x10^7$ yeast cells, then 0.5 mL CPRG was added.

Estrogenic and androgenic assay procedure. Yeast assays were carried out within a type II laminar flow. Stock solutions of chemicals were serially diluted in ethanol. Aliquots of 10 µL were then transferred 96-well optically flat-bottomed microtitre plates (Greiner Bio-One, Huber AG, Basel, Switzerland) and ethanol was allowed to evaporate to dryness. The same stock solutions were tested in both hERα and hAR assays for reliable comparison of the results. In all assays each plate contained a positive control with either E2 or DHT, in triplicates. The tested compounds were analysed in quadruplicates. A blank row with ethanol was added in order to control for a possible CPRG conversion due to the medium components or materials alone. After adding aliquots of 200 µL of the final assay medium (hERα or hAR yeast) to the plates, they were sealed with plate sealers (Micronic, Vitaris AG, Baar, Switzerland) and

shaken vigorously for 2 min on a titre plate shaker before incubation at 32 °C. The hAR plates were removed after 24 h, incubated overnight at 28°C and then at room temperature, in order to optimise the reaction without risking a significant increase in background expression of β-galactosidase. The hERα plates were removed after 72 h of incubation. This was done because

the colour change (yellow to red) occurs faster in the hAR than in the hERα assay (Sohoni and Sumpter, 1998).
After the appropriate incubation time, plates were shaken vigorously for 2 min and left for 1 h to allow yeasts to settle. The plates were than read at absorbance of 540 nm (CPRG, hormonal activity) and 620 nm (turbidity as a measure of yeast growth and as a control for cytotoxicity), using a Tecan GENios plate reader (Tecan AG, Männedorf, Switzerland).

Antiestrogenic and antiandrogenic assay procedure. Assessment of antagonistic activities was similar to agonistic activities, with the following adaptations as described previously by Sohoni and Sumpter (1998). E2 or DHT was added to the medium of the appropriate assay at a concentration that produced 65% of the maximal response (EC65), followed by the addition of the UV filter and the antagonistic standards (4HT or FT). It was measured to what extend the UV filter inhibited the colour change induced by the natural ligand.

Yeast rtERα assay

Recombinant yeast assay expressing the rainbow trout estrogen receptor alpha (rtERα assay). We investigated estrogenic activity of one selected UV filter (Et-PABA) *in vitro* by applying a quantitative β-galactosidase assay in liquid culture of recombinant yeast expressing the estrogen receptor of rainbow trout (rtERα) that was kindly provided by F. Pakdel, University of Rennes. We used a slightly modified assay procedure of the previously described assay (Petit et al., 1995; Le Guével and Pakdel, 2001). The estrogen-inducible expression system and procedure used is described in detail in (Kunz et al, 2006). Similar to the hERα, the assay is based on transactivation of rtERα and induction of β-galactosidase leading to a color change.

Preparation of rtERα assay media and yeast growth. The assay media were prepared as previously published (Kunz et al. 2006). Prior to the assay yeast cell growth was conducted as described (Petit et al., 1995), but with the modification that yeast colonies from CM medium were inoculated in Erlenmeyer flasks containing 15 mL of YPD growth medium instead of CM medium, which lead to increasing growth rates and better assay performance.

rtERα assay procedure. The whole assay was performed in clear polystyrene 96-well microplates (Greiner Bio-One, Huber AG, Basel, Switzerland) as described in detail previously

(Kunz et al., 2006). In order to assess and correct for yeast growth and as a control for cytotoxicity, yeast turbidity (A_{620}) was measured. Cytotoxicity was manifested by significantly reduced yeast growth or even cell lysis. High concentrations of some UV filters that lead to cytotoxicity were omitted from curve fitting and calculations. For screening of Et-PABA a 96-well V-bottomed microtitre plate was filled with 100 µL/well *S. cerevisiae* cells in YPD culture. Three rows contained serially diluted positive control E2, one row the ethanol-blank, and four rows the analysed UV filter in quadruplicates with increasing concentrations resulting in dose response curves. After cell lyses the lysed suspension was transferred to a new flat-bottom 96-well plate (Greiner Bio-One, Huber AG, Basel, Switzerland), ONPG was added and the estrogenic activity measured at absorbance of 405 nm as previously described (Petit et al., 1995; Le Guével and Pakdel, 2001; Schultis and Metzger, 2004).

Experiments in vivo in fish

Fish. The 14-day fish experiment was conducted using juvenile, sexually undifferentiated fathead minnows (*Pimephales promelas*), between 2-3 months of age and with a total body length between 19-27 mm. This fish species has been chosen because of its frequent used in the field of endocrine disrupters and established techniques including vitellogenin (VTG) antibodies. The experimental procedure and duration was similar to that of Panter et al. (2002)**,** who showed that estrogens are detectable after 14 days of exposure by virtue of the VTG response. Mixed sex juvenile fathead minnows were received from the cultivator (Aquatic Research Organisms, Hampton NH, USA) and adapted for a minimum of 14 d in our laboratory in aquaria prior to the experiment. Fish were fed with Tetramin pellets (Tetra GmbH, Melle, Germany) twice a day with a quantity of 1% of body weight prior to the onset of experiments. During the experiments, fish were fed with brimeshrimp (*Artemia salina*, Argent Chemical Labs, Redmond WA, USA) at a feeding rate of 1% of body weight twice a day.

Exposure. Fish were held in well-aerated reconstituted tab water medium (total hardness 160 mg/L as $CaCO_3$, total alkalinity 30 mg/L as $CaCO_3$, conductivity 500 µs/cm) and a 16 h/8 h light/dark cycle at 25±1°C. The studies were conducted using a 24 h static-renewal procedure with daily renewal of total aquaria water. For exposure, 10 randomly selected fish each were placed in stainless steel tanks (10 L) and exposed to Et-PABA at nominal concentrations of 10, 100, 500, 1000 and 5000 µg/L for 14 d. A control, solvent control (SC, 1 mL ethanol in 10 L water) and positive control for estrogenic activity (100 ng/L E2) was included. Stock solutions of Et-PABA were prepared freshly in ethanol prior to the start of the experiment and added daily to the experimental water by mixing.

Physico-chemical measurements and biological observations. Physico-chemical parameters were determined daily. pH and oxygen saturation ranged between 7.2–7.9 and 6.5–8.3 mg/

L, respectively, throughout the whole exposure period. Mortalities and abnormal behavior were recorded daily and dead fish were removed from the tanks. On day 14 all fish were anaesthesized with buffered tricaine methan sulfonate (MS-222, 100 mg/L with 200 mg $NaHCO_3$/L). Subsequently individual fish were measured, weighted, transferred into labelled Eppendorf® tubes, frozen and stored at -20 °C for homogenisation and VTG analysis.

Vitellogenin analysis. Fish were defrosted at 4°C and individually homogenized in ice-cold assay buffer (Biosense, Bergen, Norway) in a 1:2 ratio wet weight:buffer volume, using a Ultra Turax homogenizer (IKA, Huber + Co. AG, Reinach, Switzerland). The homogenates were centrifuged at 10'000 g for 3 min at room temperature using a microcentrifuge (Eppendorf centrifuge 5415 D, Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). The supernatant was withdrawn and immediately used for VTG analysis or frozen at –80 °C until required for VTG analysis. Whole-body homogenates of individual fish were assayed for VTG using a commercially available quantitative carp vitellogenin ELISA kit, which is based on a sandwich ELISA format (Biosense, Bergen, Norway) and shown to cross-react with fathead minnow VTG.

Analytical chemistry. For the duration of the experiment four aliquots of 250 mL exposure waters were taken from the two highest and the two lowest concentrations of Et-PABA and controls at the beginning (0 h) and prior to water renewal (24 h). The aliquots of the same concentration and time point were pooled in brown glass flasks, acidified with HCl to pH 2-3 and stored at 4°C until analysis. Chemical analyses of UV filter concentrations were carried out by high performance liquid chromatography and UV detection according to Kunz et al. (2006). Briefly, 25 or 250 mL of water samples, depending on sample concentration, were extracted and concentrated by solid phase extraction (SPE). The 2'500-times concentrated eluent was then analyzed by HPLC-DAD.

Data processing and statistical analysis

Recombinant yeast assays. The absorbance-measurement at 540 nm (CPRG, hERα and hAR), at 405nm (ONPG, rtERα) and 620 nm (yeast growth) allowed for subsequent correction for variations in yeast growth (turbidity), as follows:

$$\text{Corrected absorbance} = \text{chemical absorbance}_{540\,\mathrm{m}} - \text{chemical absorbance}_{620\,\mathrm{m}} - \left[\text{blank absorbance}_{540\,\mathrm{m}} - \text{blank absorbance}_{620\,\mathrm{m}}\right]$$

Concentrations of test compounds that lead to cytotoxicity, which was manifested by significantly reduced yeast growth or even cell lysis and determined by absorbance at 620 nm, were omitted from further data analysis.

For curve fitting, EC50 and IC50 calculations, the corrected absorbance values versus the logarithm of concentration were plotted, whereby the best fit from a number of non-linear regression models was selected for final data analysis. In this study, we used the four-parameter logistic equation (Hill equation) as a non-linear regression model to fit full dose-response curves according to

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(LogE50 - X) \cdot HillSlope}} \quad ,$$

where X is the logarithm of concentration and Y is the response showing a sigmoid shape. LogEC50 is the concentration of the test compound yielding half maximal effects and HillSlope is the slope parameter, which describes the steepness of the curve. A standard sigmoid dose-response curve has a Hill Slope of 1. A hill slope smaller or greater than 1, indicates a shallower or steeper curve respectively. Coefficient of determination (R^2), residuals and 95% confidence intervals were calculated so as to verify that the fitted curve represents the data correctly. The runs test was carried out in order to ensure that the model chosen to fit the curve does not significantly deviate from the data. Submaximal dose-response curves, which are defined as dose-response curves that reached only < 80% of the height of the corresponding standard, were fitted using the best fit from a number of non-linear regression models. Curve fitting was carried out using GraphPad Prism software (GraphPad Software Inc., San Diego, USA). Agonistic activities were calculated for compounds displaying full dose-response curves, which reached the same height (≥ 80% of maximal height) as the corresponding standards (maximal height = 100%). Thereby the EC50 of a compound was divided by the EC50 of E2 or DHT, respectively. For submaximal dose- response curves of lesser height than the standards (< 80%), EC50 values were calculated and used as an estimate for their agonistic activities, despite differences in curve-steepness and -height when compared to the standards. Thereby a good approximation for the E2- and DHT-equivalents for the agonistic activities of submaximal UV filters was achieved. In order to get a sufficient accurate assessment of antagonistic activities, we proceeded in the same manner as with the agonistic activities. We divided IC50 of an antiestrogenic or –androgenic compound by the IC50 of the corresponding standard (4HT or FT), and used this in the case of submaximal antagonists as approximations, because of the differences in height, shape and steepness of the dose-response curves. Efficacy, or curve height as percentage of the respective standard, was calculated as follows for all compounds:

$$Efficacy_{UVfilter} = \left[\frac{1}{Top_{Standart} - Bottom_{Standart}}\right] \cdot Top_{UVfilter} - Bottom_{UVfilter} \quad ,$$

where the curve height ($Top_{UV\ filter} - Bottom_{UV\ filter}$) of the compound was compared with the curve height of the corresponding standard ($Top_{Standard} - Bottom_{Standard,}$ set at 100 %).

Fish experiment. After testing the data distribution for normality by using the Kolmogorov-Smirnov test, means of wet weight and total length of individual fish were calculated and data analysed by analysis of variance (ANOVA) followed by a Dunnett's Multiple Comparison test to compare the treatment means with respective controls. Means of VTG concentrations of individual fish were calculated and data analysed with the non-parametric Kruskal-Wallis test followed by a Dunn's Multiple Comparison test to compare the treatment means with respective controls. Statistical comparisons with the control were made using the SC as the overall control. The results are given as mean ± standard error of mean (SEM). Differences were considered significant at $p \leq 0.05$. All computations were performed with PRISM 4.0 (GraphPad Software Inc., San Diego, USA).

Results

We have systematically analysed 18 commercially available and commonly used UV filters for estrogenic, antiestrogenic, androgenic and antiandrogenic activities *in vitro*. 4HB was also assessed for these activities, a compound which is not used as an UV filter itself, but represents an estrogenic metabolite. The recombinant yeast assays, carrying either hERα or hAR, were used for assessing either estrogenic and antiestrogenic, or androgenic and antiandrogenic activities, respectively (Routledge and Sumpter, 1996; Routledge and Sumpter, 1997; Sohoni and Sumpter, 1998). The hERα assay showed a high degree of specificity for estrogens, whereas the hAR assay was not similarly specific for androgens. E2 was able to stimulate β-galactosidase activity in the hAR assay, but was 20-times less potent than DHT (data not shown), as demonstrated previously for the hAR assay (Routledge and Sumpter, 1996; Gaido et al., 1997; Routledge and Sumpter, 1997; Sohoni and Sumpter, 1998). In every assay we checked for potential cytotoxicity caused by the test compounds, by routinely measuring yeast

Table 2. Cytotoxicity of investigated UV filters in hERα and hAR transactivation assays.

Compound	hERα assay Cytotoxicity (M)	hAR assay Cytotoxicity (M)
4MBC	--	--
3BC	--	$>3.3x10^{-4}$
BP1	--	$>2.0x10^{-5}$
BP2	$>2.5x10^{-3}$	$>3.1x10^{-4}$
4HB	$>5.0x10^{-4}$	$>4.2x10^{-5}$
4DHB	--	--
BP3	--	--
BP4	--	--
IMC	--	--
OMC	--	--
OC	--	--
BS	--	--
PS	--	--
HMS	--	--
OS	--	--
PABA	--	--
Et-PABA	$>2.5x10^{-3}$	$>2.5x10^{-3}$
OD-PABA	$>2.5x10^{-3}$	--
PEG25-PABA	--	--

Abbreviations of compounds see Table 1. --, no cytotoxicity; Cytotoxic concentrations derive from 3 experiments with four replicates each.

growth (620 nm) besides β-galactosidase activity (540 nm). Hence test compound concentrations, which lead to significant reduced yeast cell growth or complete growth inhibition, were omitted from data analysis for hormonal activities. As shown in Table 2, cytotoxicity was found for 3BC, BP1, BP2, 4HB and OD-PABA at high concentrations. As an example cytotoxicity and normal variations in yeast growth are shown for the antiandrogenic compounds in Figure 1. Hormonal activity was therefore assessed at non-cytotoxic concentrations only.

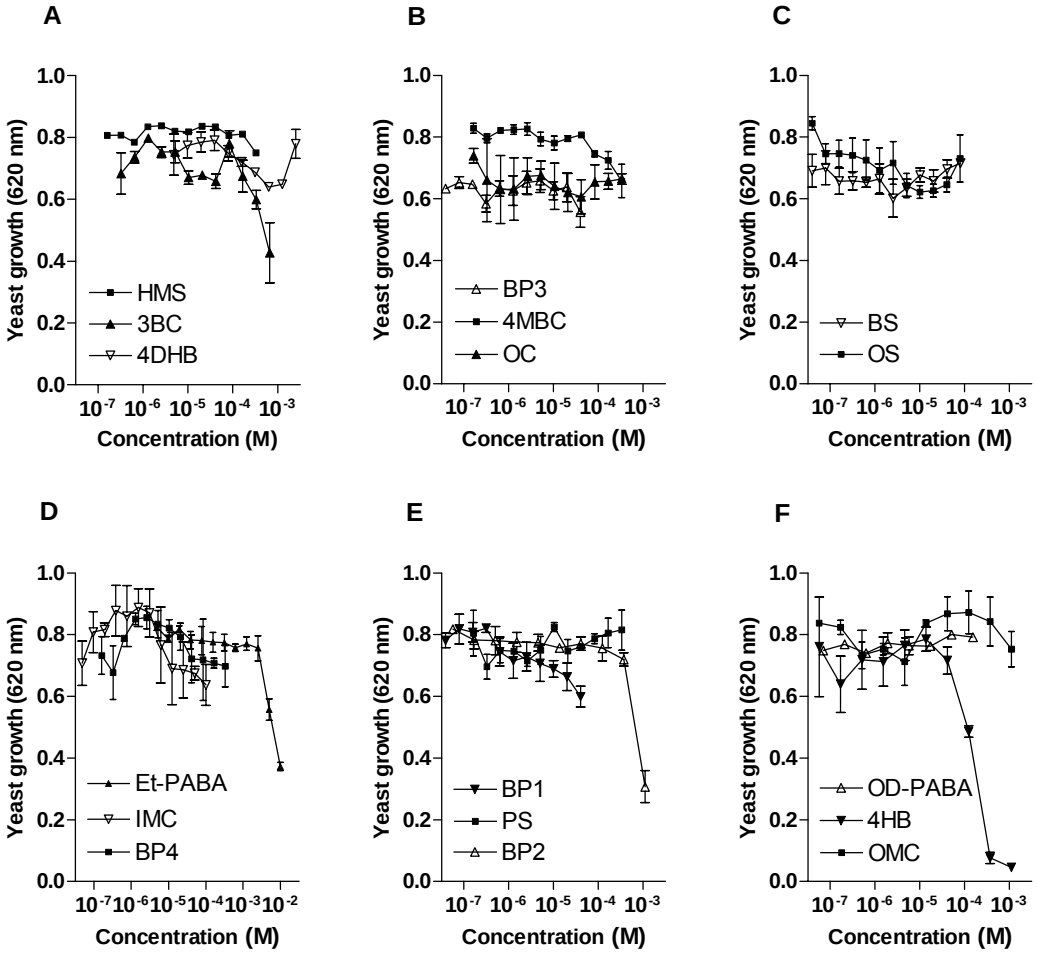


Figure 1. Yeast growth and cytotoxicity of antiandrogenic compounds in the hAR assay, shown in six panels (A to F) for clarity. Data represent. 3 experiments with 4 replicate each and are shown in means ± S.E.M.

Estrogenic activity of UV filters in fish in vitro

Estrogenic activity. Nine of 18 analysed UV filters, and the 4HB metabolite exhibited estrogenic activity in the hERα assay. BP1, BP2, 4DHB, 4HB and Et-PABA were full hERα agonists exhibiting full dose-response curves (Fig. 2A). The most potent UV filter tested was BP1, which was found to be about 5'000 times less potent than E2. 4HB, BP2, 4DHB and Et-PABA showed lower estrogenicity being approximately 16'000-, 21'000-, 170'000 and 3'500'000-times less potent than E2, respectively (Tab. 3). Submaximal dose-response curves were observed for

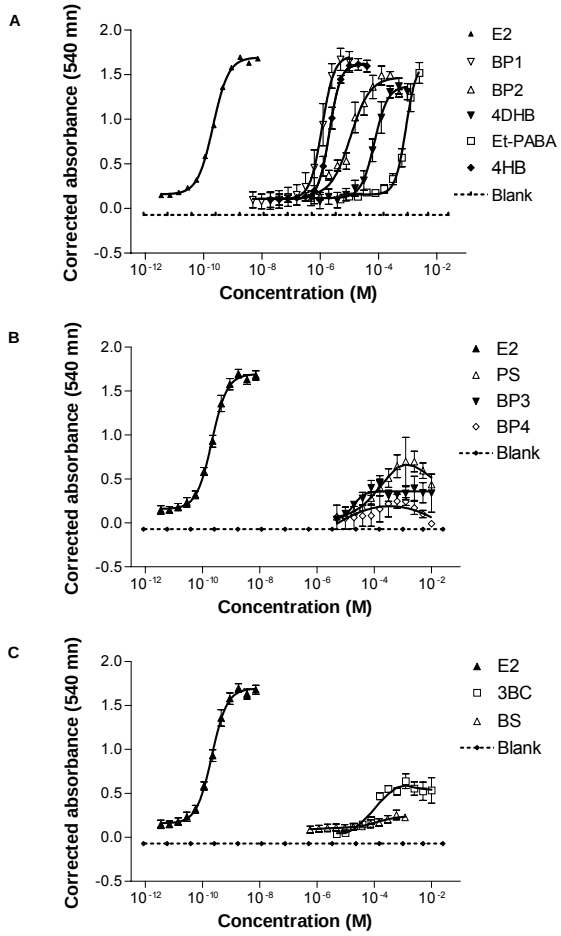


Figure 2. Estrogenic compounds in the hERα assay, shown in panel A, B and C for clarity. Data shown are means ± S.E.M. (3 experiments with 4 replicates each).

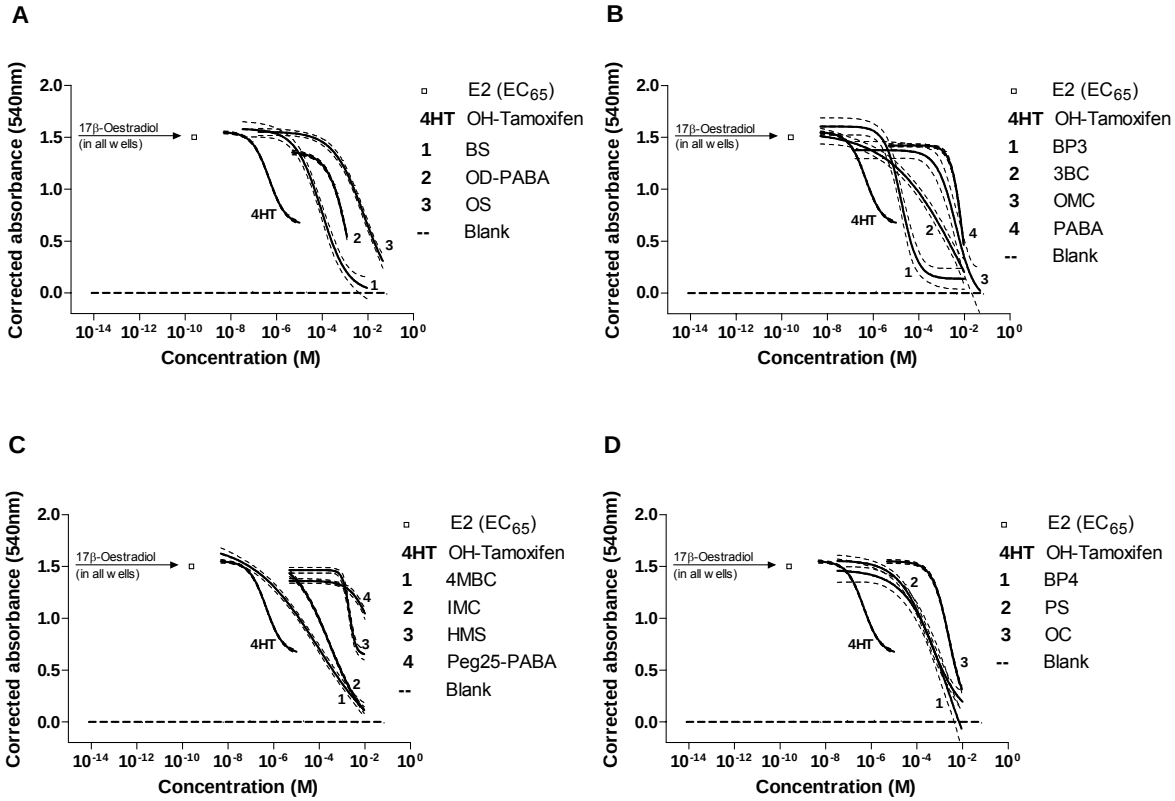


Figure 3. Antiestrogenic compounds in the hERα assay, shown in four panels (A, B, C and D) for clarity. Data shown are means and 95% confidence intervals (3 experiments with 4 replicates each).

Table 3. Effects of 18 UV filters and E2 on the estrogenic response in the yeast hERα trans-activation assay.

Compound	Efficacy[a]	Estrogenic activity[b]	Asymmetric hill function parameters		
			Hill Slope	Max[c]	EC50 (M)
E2	100%	1	1.632 ± 0.38	1.536 ± 0.16	2.59E-10 ± 1.19E-10
4HT	nd	-	-	-	nd
4MBC	nd	-	-	-	nd
3BC	21%	*1/1'300'000*	1.000	0.326	3.10E-04
BP1	96%	1/5'000	1.300	1.604	1.15E-06
BP2	91%	1/21'000	2.107	1.336	1.09E-05
4HB	108%	1/16'000	1.794	1.717	1.82E-06
4DHB	91%	1/170'000	1.902	1.256	7.34E-05
BP3	18%	*1/45'000*	2.322	0.324	1.86E-05
BP4	6%	*1/380'000*	1.000	0.097	9.48E-05
IMC	nd	-	-	-	nd
OMC	nd	-	-	-	nd
OC	nd	-	-	-	nd
BS	12%	*1/860'000*	1.000	0.157	1.66E-04
PS	32%	*1/480'000*	1.699	0.529	1.10E-04
HMS	nd	-	-	-	nd
OS	nd	-	-	-	nd
PABA	nd	-	-	-	nd
Et-PABA	87%	3'515'963	2.395	1.490	$8.69x10^{-4}$
OD-PABA	nd	-	-	-	nd
PEG25-PABA	nd	-	-	-	nd

Abbreviations: For abbreviations of listed compounds see Table 1, except E2, 17β-estradiol; 4HT, 4-hydroxytamoxifen; nd, not detected; -, not calculated; EC50, the concentration of the compound exhibiting 50% of its total effect; [a] effect (curve height) of a compound given as percentage of the effect of E2; [b] ratio of the EC50 of a compound divided by the EC50 of the corresponding assay standard, numbers in italics are approximations for compounds with submaximal activity; [c] curve-height = top – bottom; Values for E2-standard are given in mean S.E.M. (n= 9). Value of compounds from 3 experiments with four replicates each.

Table 4. Effects of 18 UV filters and 4HT on the antiestrogenic response in the yeast hERα transactivation assay.

Compound	Efficacy[a]	Antiestrogenic activity[b]	Asymmetric hill function parameters		
			Hill Slope	Max[c]	IC50 (M)
4HT	100%	1.000	-1.199 ± 0.28	0.913 ± 0.11	4.92E-07 ± 2.14E-07
4MBC	181%	*1 /190*	-0.300	1.596	8.73E-05
3BC	134%	*1/23'500*	-0.284	1.301	8.46E-03
BP1	nd	-	-	-	nd
BP2	nd	-	-	-	nd
4DHB	nd	-	-	-	nd
4HB	nd	-	-	-	nd
BP3	168%	*1/45*	-1.226	1.471	1.78E-05
BP4	188%	*1/5'700*	-0.495	1.518	2.17E-03
IMC	126%	*1/540*	-0.539	1.330	2.97E-04
OMC	182%	*1/14'900*	-1.049	1.368	4.30E-03
OC	118%	1/2'250	-1.214	1.248	2.57E-03
BS	178%	*1/195*	-0.765	1.541	9.45E-05
PS	156%	*1/1'000*	-0.611	1.368	3.72E-04
HMS	98%	1/3'500	-3.074	0.817	2.06E-03
OS	164%	*1/20'000*	-0.649	1.274	6.79E-03
PABA	98%	1/24'300	-1.685	0.959	9.23E-03
Et-PABA	nd	-	-	-	nd
OD-PABA	78%	1/4'600	-0.880	0.844	2.95E-03
PEG25-PABA	37%	*1/75'500*	-0.910	0.362	3.71E-02

Abbreviations: For abbreviations of listed compounds see Table 1; nd, not detected; -, not calculated;IC50, concentration of compound exhibiting 50% of its total possible inhibition; [a] effect (curve height) of a compound given as percentage of the effect of 4HT; [b] ratio of the IC50 of a compound divided by the IC50 of the corresponding assay standard, numbers in italics are approximations for compounds with submaximal activity; [c] curve-height = top – bottom; Values for 4HT-standard are given in mean S.E.M. (n= 14). Value of compounds from 3 experiments with four replicates each.

BP3, BP4, BS, PS and 3BC indicating partial agonism to hERα (Fig. 2B-C). These UV filters displayed estrogenicity between 45'000- and 1.3 million-times lower than E2 and exhibited efficacies between 6-32 % (Tab. 3). The remaining 9 UV filters were not estrogenic up to 10^{-2} M (data not shown).

Antiestrogenic activity. A surprisingly high number of 14 UV filters inhibited the activity of E2 (EC65) in the hERα assay (Fig. 3), thus demonstrating antiestrogenicity. Noteworthy, the 13 UV filters 4MBC, 3BC, BP3, BP4, IMC, OMC, HMS, OC, BS, PS, OS, PABA and OD-PABA completely inhibited the activity of E2 at the highest concentrations tested and produced full dose-response curves. This indicates a much higher frequency of antiestrogenic than estrogenic activity. PEG25-PABA leads to submaximal inhibition at highest concentrations only. The most potent antiestrogenic UV filter tested was BP3 being about 45-times less potent than the known antiestrogen 4HT. 4MBC, BS and IMC were 190- to 540-times less potent and the other

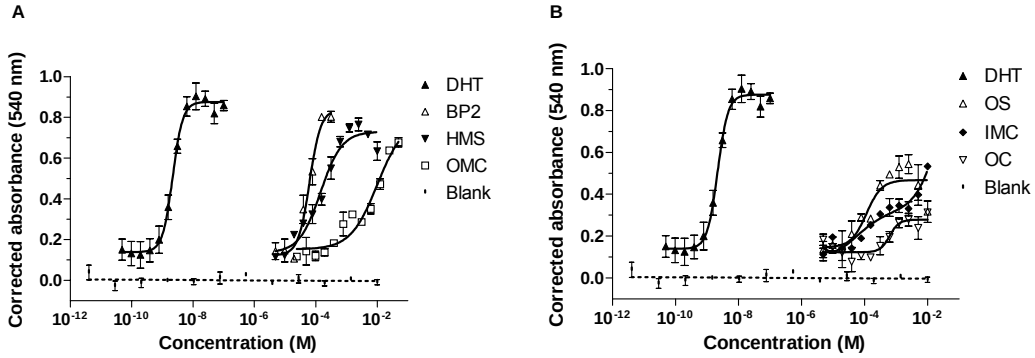


Figure 4. Androgenic compounds in the hAR assay, shown in panel A and B for clarity. Data are shown as means ± S.E.M. (3 experiments with 4 replicates each).

antagonistic UV filters were between 1'000- and 25'000-times less potent than 4HT (Tab. 4). PEG25-PABA, the only UV filter with submaximal dose-response curve and an efficacy of 37%, showed a 75'000-times lower antiestrogenic activity (Tab. 4).

Androgenic activity. Six UV filters were found to possess androgenic activity in the hAR assay (Fig. 4). BP2 and HMS produced full dose-response curves, while OMC, OS, OC and IMC displayed a partly agonistic behaviour indicated by submaximal dose-response curves. BP2 was the most active androgenic UV filter being about 30'000-times less potent than DHT. HMS was approximately 110'000-times less potent (Tab. 5). UV filters with submaximal activities reached efficacies between 21-61%. OS was found to be 45'000-times, IMC 260'000-times, OC 290'000-times and OMC 4'100'000-times less potent than DHT (Tab.5).

Antiandrogenic activity. A surprisingly high number of UV filters inhibited androgenic activity, demonstrating antiandrogenicity. Of the 19 compounds analysed, 16 UV filters and the metabolite 4HB displayed full dose-response curves with complete inhibition of DHT activity

(Fig. 5). This was found with 4MBC, 3BC, BP1, BP2, BP3, BP4, 4DHB, IMC, OMC, HMS, OC, BS, PS, OS, PABA and 4HB. Four UV filters produced non-monotonic dose-response curves. PS displayed antagonistic activity at low concentrations, followed by an agonistic interaction with DHT at medium concentrations and again antagonism at high concentrations. BP2 followed the same pattern as PS, except of cytotoxicity at high concentrations (>3.7x10^{-4} M). OD-PABA displayed a rather hyperbola-like inhibition of DHT, whereas 4DHB showed a sigmoidal curve at low concentrations followed by a weak androgenic effect at medium to

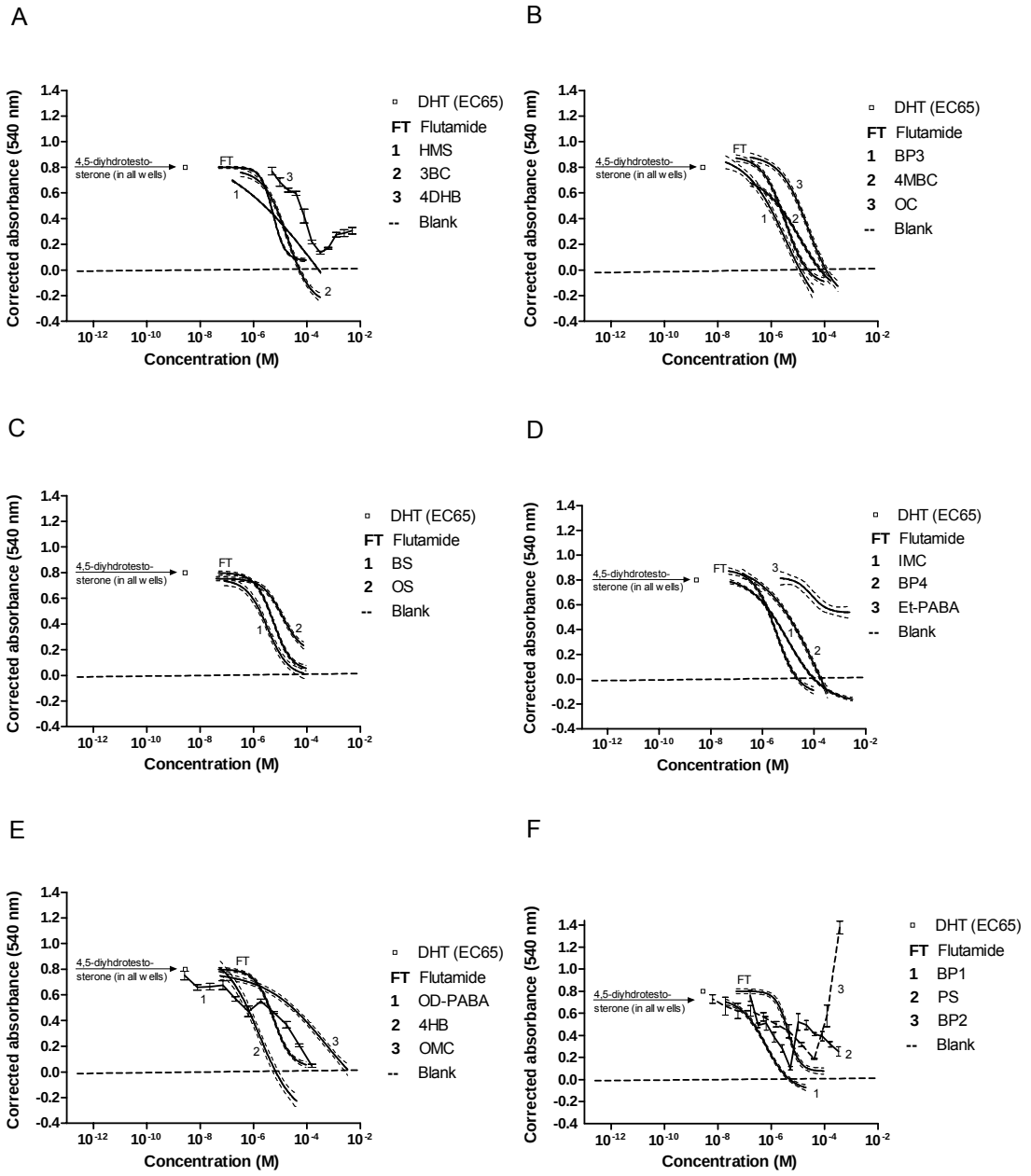


Figure 5. Antiandrogenic compounds in the hAR assay, shown in six panels (A to F) for clarity. Data are shown in means and 95% confidence intervals, except for non-monotonic curves (mean ± S.E.M.). Data are representative of 3 experiments with 4 replicates each.

Table 5. Effects of 18 UV filters and DHT on the androgenic response in the yeast hAR transactivation assay.

Compound	Efficacy[a]	Androgenic activity[b]	Asymmetric hill function parameters		
			Hill Slope	Max[c]	EC50 (M)
DHT	100%	1	2.707 ± 1.13	0.724 ± 0.18	2.07E-09 ± 3.89E-10
FT	nd	-	-	-	nd
4MBC	nd	-	-	-	nd
3BC	nd	-	-	-	nd
BP1	nd	-	-	-	nd
BP2	99%	1/30'000	2.349	0.714	6.27E-05
4HB	nd	-	-	-	nd
4DHB	nd	-	-	-	nd
BP3	nd	-	-	-	nd
BP4	nd	-	-	-	nd
IMC	52%	*1/260'000*	1.000	0.310	4.29E-04
OMC	61%	*1/4'100'000*	1.000	0.635	1.01E-02
OC	21%	*1/290'000*	2.946	0.155	6.27E-04
BS	nd	-	-	-	nd
PS	nd	-	-	-	nd
HMS	104%	1/110'000	1.468	0.545	1.70E-04
OS	44%	*1/45'000*	1.793	0.321	1.14E-04
PABA	nd	-	-	-	nd
Et-PABA	nd	-	-	-	nd
OD-PABA	nd	-	-	-	nd
PEG25-PABA	nd	-	-	-	nd

Abbreviations: For abbreviations of listed compounds see Table 1, except DHT 4,5-dihydrotestosterone; FT, flutamide; nd, not detected; -, not calculated; EC50, concentration of compound exhibiting 50% of its total effect; [a] effect (curve height) of a compound given as percentage of the effect of DHT; [b] ratio of the EC50 of a compound divided by the EC50 of DHT, numbers in italics are approximations for compounds with submaximal activity; [c] curve-height = top – bottom; Values for DHT-standard are given in mean S.E.M. (n= 6). Value of compounds from 3 experiments with four replicates each.

Table 6. Effects of 18 UV filters and FT on the antiandrogenic response in the yeast hAR transactivation assay.

Compound	Efficacy[a]	Antiandrogenic activity[b]	Asymmetric hill function parameters		
			Hill Slope	Max[c]	IC50 (M)
FT	100%	1	-1.474 ± 0.62	0.625 ± 0.13	4.32E-06 ± 1.81E-06
4MBC	107%	*1/3*	-0.621	0.558	1.18E-05
3BC	143%	*1/3*	-0.989	0.625	1.85E-05
BP1	107%	*4*	-0.937	0.471	6.92E-07
BP2	79%	*1.3*	non-sigmoidal dose-response-curve		
4HB	155%	*2.5*	-0.676	0.489	1.69E-06
4DHB	94%	*1/11*	non-sigmoidal dose-response-curve		
BP3	115%	1/1.3	-0.581	0.507	3.68E-06
BP4	108%	*1/40*	-0.420	0.663	1.59E-04
IMC	96%	*1/3.5*	-0.683	0.516	8.12E-06
OMC	123%	*1/37*	-0.504	0.853	3.12E-04
OC	86%	1/11	-0.825	0.582	2.45E-05
BS	101%	1.2	-0.358	0.750	2.58E-06
PS	75%	*1.7*	non-sigmoidal dose-response-curve		
HMS	104%	*1/17*	-0.324	0.621	1.07E-04
OS	77%	*1/1.1*	-0.839	0.810	7.34E-06
PABA	nd	-	-	-	nd
Et-PABA	34%	*1/87*	-0.440	0.198	2.65E-04
OD-PABA	102%	*1/2*	non-sigmoidal dose-response-curve		
PEG25-PABA	nd	-	-	-	nd

Abbreviations: For abbreviations of listed compounds see Table 1, except FT, flutamide; nd, not detected; -, not calculated; Submax, submaximal dose response curve; IC50, concentration compound exhibiting 50% of its total possible inhibition. ; [a] effect (curve height) of a compound given as percentage of the effect of FT; [b] ratio of the IC50 of a compound divided by the IC50 of FT, numbers in italics are approximations for compounds with submaximal activity; [c] curve-height = top – bottom; FT-standard as mean ± S.E.M. (n= 16). Value of compounds from 3 experiments with four replicates each.

Table 7. Semiquantitative assessment of the agonist and antagonist activities of the compounds in the hERα and hAR assay.

Compound	Estrogenic activity	Anti-estrogenic activity	Androgenic activity	Anti-androgenic activity
4-Methylbenzylidene camphor (4MBC)	--	+++	--	**+++**
3-Benzylidene camphor (3BC)	+	+++	--	**+++**
Benzophenone-1 (BP1)	+++	--	--	**+++**
Benzophenone-2 (BP2)	+++	--	+++	**+++**
4-Hydroxy benzophenone (4HB)	**+++**	--	--	**+++**
4,4'-Dihydroxybenzophenone (4DHB)	**+++**	--	--	**+++**
Benzophenone-3 (BP3)	+	+++	--	**+++**
Benzophenone-4 (BP4)	**+**	+++	--	+++
Isopentyl-4-methoxycinnamate (IMC)	--	+++	++	**+++**
Octyl methoxycinnamate (OMC)	--	+++	++	**+++**
Octocrylene (OC)	--	+++	+	**+++**
Benzyl salicylate (BS)	+	**+++**	--	+++
Phenly salicylate (PS)	++	+++	--	**+++**
Homosalate (HMS)	--	+++	+++	**+++**
Octyl salicylate (OS)	--	+++	++	**+++**
Para amino-benzoic acid (PABA)	--	+++	--	--
Ethyl-4 amino benzoate (Et-PABA)	**+++**	--	--	++
Octyl dimethyl para amino benzoate (OD-PABA)	--	+++	--	**+++**
Ethoxylated ethyl 4-amino benzoate (Peg25-PABA)	--	+	--	--

Abbreviations: Signs indicate efficacy of each compound in different assays: +++, maximal dose-response curves with ≥ 80% efficacy; ++, submaximal dose-response curves with ≥ 30% efficacy; +, submaximal dose-response curves with < 30% efficacy. Bold, most potent hormonal activity found for each compound; --, not detected.

high concentrations. BP1 was the most potent antiandrogenic UV filter tested and even found to be about 4-fold more potent than the known antiandrogen FT. 4HB was approximately 2.5- fold more potent than FT, whereas PS, BP2, BS, OS, and BP3 were about equally potent (Tab. 6). OD-PABA, 4MBC, 3BC and IMC were 2- to 3-times weaker than FT and the other antiandrogenic compounds showed between 11- and 40-times lower antiandrogenic activities (Tab. 6). Et-PABA was the only antiandrogenic UV filter, which only partially inhibited DHT and it was cytotoxic at high concentrations (>$2.5x10^{-3}$ M).

Multiple hormonal activities of UV filters. To our surprise all investigated UV filters possessed hormonal activities *in vitro*, as demonstrated by the relative potencies of the chemicals in the different assays (Tab. 7). Moreover, most of the UV filters showed multiple hormonal activities.

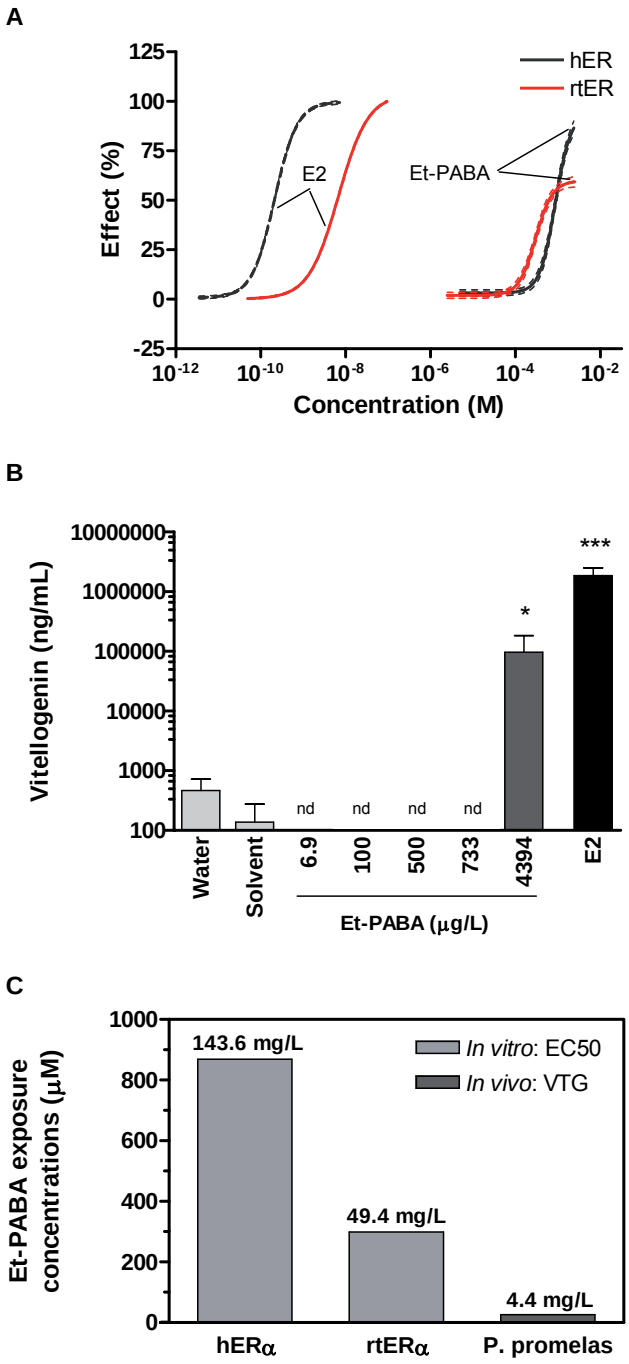


Figure 6. (A) Comparison of estrogenic activity of Et-PABA between the rtERα (bold lines) and the hERα (dashed lines) assay. Data shown are means and 95% confidence intervals (3 experiments with 4 replicates each). Effect (%) represents percentage of β-galactosidase induction of UV filters versus solvent (0%) and E2 (100%) controls. Compound abbreviations see Table 1. (B) Vitellogenin concentration in juvenile fathead minnows exposed to eight UV filters. Values are means ± SEM (n=10). Asterics denote a significant difference from solvent control at $p \leq 0.05$. Concentrations given as actual median measured, except 100 and 500 µg/L. nd, no VTG induction was observed. (C) Comparison of in vitro (rtERα, hERα) and in vivo effect concentrations. Abbreviations: E2, 17β-estradiol; EC50, the concentration of the compound exhibiting 50% of its total effect. Values for E2-standard are given in mean S.E.M. of 10 (rtERα) or 9 (hERα, Kunz et al, 2006) experiments, respectively, with three replicates each. EC50 values of compounds are from 3 experiments with four replicates each. Potency = EC50 compound/EC50 E2 is calculated from mean values of each single experiment; the used EC50 values for E2 in each experiment are not listed here for simplicity reasons. Chemical concentrations shown for the in vivo assay are those at which VTG was significantly induced relative to the solvent control. For compound abbreviations see Table 1.

Surprisingly, 11 compounds displayed as much as three distinct hormonal activities each. All compounds were full hAR antagonists, except Et-PABA with partial antiandrogenic activity, and PABA and PEG25-PABA with no antagonistic activity. Hence antiandrogenicity and estrogenicity were either found along with androgenic activity (BP2) or with antiestrogenic (3BC, BP3, BP4, BS and PS). Moreover antiandrogenicity was found to be coupled with antiestrogenic and androgenic activities (IMC, OMC, HMS, OC and OS). Five compounds exhibited two distinct hormonal activities each, while antiandrogenicity was either coupled with antiestrogenicity or estrogenicity, which holds for 4MBC, BP1, 4HB, 4DHB, Et-PABA and OD-PABA. Interestingly, full hERα agonists did not possess any antiestrogenic activity, whereas partial hERα agonists

and compounds with no estrogenic activity were fully antiestrogenic (except PEG25-PABA, displaying submaximal antiestrogenicity). For androgenic and antiandrogenic compounds no such pattern was observed (PABA and PEG25-PABA displayed antiestrogenicity only).

Comparison with rtERα. Et-PABA that was found estrogenic in the yeast hERα assay (Fig. 2) was investigated for its estrogenicity in a recombinant yeast system expressing the rainbow trout estrogen receptor alpha (rtERα). The estrogenic activity of Et-PABA was relatively higher with rtERα than hERα, but with a lower efficacy in the rtERα assay, where Et-PABA produced a submaximal dose response curve only (Fig. 6 A). Et-PABA displayed an estrogenic activity of 38'547-times less than that of E2 with rtERα and 3'515'963-times less with hERα. In contrast to the relatively higher activity of Et-PABA in the rtERα assay, E2 was about 62-times more active in the hERα assay.

Estrogenic activity of UV filters in fish in vivo

Measured exposure concentrations. Concentrations of Et-PABA in aquaria waters was measured at the beginning of exposures (0 h) and 24 h later prior to water renewal at the lowest and the two highest exposure concentrations in order to determine actual effect concentrations and to get an estimate of concentration decrease. At 0 h actual concentrations determined by HPLC analysis were close to nominal; they were 9.2 µg/L (nominal 10 µg/L), 921 µg/L (nominal 1000 µg/L), and 4'939 µg/L (nominal 5000 µg/L), respectively. After 24 h before water renewal, concentrations decreased to 45-77% of initial values and were 4.5 µg/L (45%, nominal 10 µg/L), 595 µg/L (60%, nominal 1000 µg/L), and 3'848 µg/L (77%, nominal 5000 µg/L), respectively.

Estrogenicity of Et-PABA. No mortality was observed in controls, solvent controls (SC) and positive controls (E2) exposed fish. Et-PABA did not affect survival during exposure. No difference occurred in wet weight and mean length in the SC and E2, and no decreases in body weight gain and body length were observed for Et-PABA at all exposure concentrations.
Et-PABA, which exhibited estrogenic activity *in vitro* was also estrogenic *in vivo* at high concentrations (Fig. 6). VTG induction was significant at the highest exposure concentration of 4'919 µg/L Et-PABA (96'281 ng VTG/mL, $p < 0.05$). Significant VTG induction also occurred in fish exposed to the 100 ng/L E2 (positive control). Mean whole-body VTG content was 2'600'000 ng/mL and highly induced ($p < 0.001$), compared to the water and solvent control having a residual level of 300 ng/mL.

Discussion

Our study reveals a more detailed picture of the hormonal activity of UV filters *in vitro* and *in vivo* and discloses an unexpected complexity of their multiple hormonal properties. Until recently estrogenicity was regarded as the most important endocrine activity of UV filters *in vitro* (Miller et al., 2001) and *in vivo* in rats and fish (Schlumpf et al., 2001; Schlumpf et al., 2004; Kunz et al., 2006). To our surprise all investigated compounds were found to possess hormonal activity either in the hERα or hAR assay, or both. For one UV filter, we compared the activity in the hERα assay with that in the rtERα assay and found that Et-PABA was equally estrogenic in both assays despite the lower activity of E2 in the rtERα assay. In fish we observed that this UV filer was estrogenic *in vivo* as well. This clearly demonstrates that Et-PABA leads to transactivation of both the hERα and rtERα in recombinant yeasts. Both *in vitro* systems are indicative for estrogenicity *in vivo*, but the rtERα had a more predictive power for *in vivo* activity in fish. Hence estrogenic activity of chemicals is best assessed by the use of a tiered approach with a combination of *in vitro* and *in vivo* assays incorporating related or even identical species. Our findings may be of considerable practical importance due to the occurrence of UV filters residues in aquatic ecosystems and in fish (Balmer et al., 2005; Buser et al., 2006), caused by frequent use of these compounds in sunscreens, skin care products, cosmetics and a variety of different materials.

hERα agonistic and antagonistic UV filters. According to their estrogenic and antiestrogenic activities observed in the hERα assay, UV filters can be classified into three groups. First, compounds such as the benzophenones, BP1, BP2, 4HB and 4DHB and the para amino benzoate Et-PABA, with pure estrogenic activity showing full dose-response curves. Second, compounds including BP3, BP4, BS, PS, and 3BC with submaximal estrogenic activity and full antiestrogenic activity. Third, pure antiestrogenic compounds lacking any estrogenic activity including 4MBC, IMC, OMC, OC, HMS, OS, PABA, OD-PABA, and PEG25-PABA.

Nine UV filters and the metabolite 4HB exhibited estrogenic activity in the hERα assay, either as full or partial hERα agonists. Even though all benzophenones were estrogenic, partly because they were carrying at least one ring substituted hydroxyl group, full estrogenic activity was only exhibited when the OH-group was situated in *para*- or, *ortho*- and *para*-position (BP1, 4HB, BP2, 4DHB). Of these full agonists the highly symmetric benzophenones (4DHB and BP2) possessed weaker estrogenic activity than their asymmetric counterparts (4HB and BP1). Et-PABA, the weakest of the pure agonists, has only one benzene ring, a polar amino-group it attached to it, which might be the reason for its maximal estrogenic activity (Routledge and Sumpter, 1997; Schultz et al., 2000).
These structural features required for optimal estrogenic activity, were already described for alkylphenols (Routledge and Sumpter, 1997; Sohoni and Sumpter, 1998; Schultz et al., 2000)

and match with previous results for benzophenones (Schultz et al., 2000). Furthermore, these UV filters lack any antiestrogenic activity and even enhanced E2 activity; hence they act as pure hERα agonist.

In contrast, a second group of UV filters displayed only submaximal estrogenic activity and completely inhibited the activity of E2 in the hERα assay. This partial hERα agonism such as of BP3 and BP4 may be caused by additional substituents like hydroxyl-, methoxy- or sulfonic acid groups. This was reported for benzophenones (Miller et al., 2001), but also found in our study for the salicylates BS and PS, and the camphor derivate 3BC.
Partial agonism of a compound is characterized by its reduced efficacy, eliciting only submaximal dose-response curves and its ability to block the effect of full agonist when co-exposed with this compound. If a partial agonist is exposed alone or in the presence of low concentrations of a full agonist, the resulting effect is submaximal agonistic or additive, respectively (for details see Stephenson (1997) and Kenakin (2004)). We hypothesize that partial agonistic UV filters of the second group preferably bind to the hormone-binding site of hERα and display a similar behavior as proposed for type I antiestrogens, which are characterized by partial agonistic activity at low concentrations and antagonistic activity at high concentrations (Jensen and Khan, 2004). Exposed alone these UV filters display submaximal estrogenic activity only, whereas co-exposure with high concentrations of the full hER-agonist E2 results in antiestrogenic activity. A possible mode of action that accounts for partial agonism of UV filters is that they only induce a suboptimal conformational change of the hERα, which needs a agonist induced conformational change to be activated. Thus partial agonists can lead to steric or ionic interferences, which then result in reduced efficacy (Pike et al., 1999; Bowers et al., 2000; Gangloff et al., 2001). Another possible mode of action is the two site model, that showed that type I antiestrogens have a higher affinity for the hormone (E2-specific) than for a secondary (antagonist-specific) binding site (Dudley et al., 2000; Jensen and Khan, 2004). Studies with 4HT or tamoxifen indicate that the hERα may indeed have more than one binding site, whereby these type I antiestrogens were shown to act primarily through the hormone-binding site of the hERα but are also able to bind to a secondary binding site (Dudley et al., 2000).

On the other hand, a third arbitrary group of compounds lacked estrogenic activity and had, with very few exceptions, only one non-hydroxylated ring that possessed other substituents such as ethoxy, alkyl-, amino-, cyano- or methoxy-groups, which were shown to significantly decrease the chemical's affinity for the estrogen receptor (Blair et al., 2000). 4MBC, IMC, OMC, OC, HMS, OS, PABA, and OD-PABA acted as pure hERα antagonists inhibiting E2 dose-dependently, like type II antiestrogens such as ICI 182780 (Dudley et al., 2000). Possibly, antiestrogenic UV filters bind to and block the primary E2 binding site of the hERα, and it is assumed that they are able to bind with comparable affinities to a secondary binding site of the

Table 8. Agonist and antagonist activities of UV filters found in other in vitro and in vivo assays.

	Hormonal activities			
	Estrogenic	Antiestrogenic	Androgenic	Antiandrogenic
4MBC	*In vitro,* Schlumpf et. al (2004) *In vivo,* Schlumpf et al. (2004) *In vivo,* Müller et al. (2003) *In vivo,* Tinwell et al. (2002) *In vivo,* Inui et al. (2003)	*In vitro,* Müller et al. (2003)	--	*In vitro,* Ma et al. (2003)
3BC	*In vitro*, Yamasaki 2003 *In vivo,* Schlumpf et al. (2004) *In vivo*, Müller et al. (2003) *In vivo*, Tinwell et al. (2002) *In vivo*, Holbech et al. (2002) *In vivo*, Kunz et al. (2006)	--	--	*In vitro*, Ma et al. (2003)
BP1	*In vitro*, Yamasaki 2003 *In vivo*, Schlumpf et al. (2004) *In vivo*, Müller et al. (2003) *In vivo*, Tinwell et al. (2002) *In vivo*, Kunz et al. (2006)	--	--	--
BP2	*In vitro*, Yamasaki 2003 *In vivo*, Schlumpf et al. (2004) *In vivo*, Müller et al. (2003) *In vivo*, Tinwell et al. (2002) *In vivo*, Kunz et al. (2006)	--	--	--
4DHB	*In vivo*, Schlumpf et al. (2004) *In vivo*, Müller et al. (2003) *In vivo*, Tinwell et al. (2002)	--	--	--
BP3	*In vitro*, Yamasaki 2003 *In vivo*, Schlumpf et al. (2004) *In vivo*, Müller et al. (2003) *In vivo*, Tinwell et al. (2002)	*In vivo*, Müller et al. (2003)	--	*In vitro*, Ma et al. (2003)
BP4	--	--	--	*In vitro*, Ma et al. (2003)
OMC	*In vivo*, Schlumpf et al. (2004) *In vivo*, Müller et al. (2003) *In vivo*, Tinwell et al. (2002) *In vivo*, Inui et al. (2003)	--	--	--
HMS	--	*In vitro*, Müller et al. (2003)	--	--
OD-PABA	--	--	--	*In vitro*, Ma et al. (2003)

Abbreviations: For abbreviations of compounds see Table 1. For full references of the cited literature see the referenc list. --, no data available.

receptor (Collins et al., 1997; Jensen and Khan, 2004). They cause a conformational change (Hedden et al., 1995), interfere with hERα dimerisation and result in antagonism. PEG25-PABA displayed submaximal antiestrogenicity only, probably because of the ethoxylated side-chains, resulting in a large molecule that might only partly be able to sterically hinder E2 binding.

Estrogenicity of UV filters - comparison with other in vitro and in vivo systems. Our results are consistent with other *in vitro* studies using the same system (Miller et al., 2001) or one expressing a rainbow trout estrogen receptor (Schlumpf et al., 2004; Kunz et al., 2006) (for details see Tab. 8). BP1, BP2, BP3 and 3BC were estrogenic in MCF-7 cells (Schlumpf et al., 2001; Schlumpf et al., 2004) and reporter hERα /HeLa cells (Yamasaki et al., 2003). 4MBC lacked estrogenicity in the hERα assay (our data and Tinwell et al. (2002), but induced MCF-7 cell proliferation (Schlumpf et al., 2004). *In vivo* BP1, BP2, 4DHB, BP3, 3BC, 4MBC and OMC were shown to have estrogenic activity in rats (Tinwell et al., 2002; Mueller et al., 2003; Schlumpf et al., 2004) and in fish. 4MBC and OMC were estrogenic in juvenile rainbow trout (Inui et al., 2003) and 3BC in medaka (Holbech et al., 2002) and BP3 in rainbow trout (Daniel Schlenk, personal communication), whereas no estrogenicity was observed for OMC, OD-PABA, HMS, BP3 and 4MBC in zebrafish (Schreurs et al., 2002). In fathead minnows we found that 3BC, BP1 and BP2 lead to vitellogenin induction (Kunz et al., 2006). At environmental concentrations, however, neither 4MBC, nor 3BC showed estrogenic or androgenic activity such as skewed sex-ratios in tadpoles of frogs (Kunz et al., 2004). However, environmental samples of marine sediment and New York City wastewater, which also contained the UV filter BP3 were found to be estrogenic in rainbow trout and male medaka (Sapozhnikova et al., 2005; Schlenk et al., 2005).

Our identification of 13 antiestrogenic UV filters in the hERα assay is consistent with the recently reported *in vitro* antiestrogenicity of 4MBC, HMS and BP3 (Mueller et al., 2003). In human Ishikawa cells, 4MBC inhibited E2 binding at the ERα and ERβ (Mueller et al., 2003), whereas in the HEK 293 reporter gene system, neither 4MBC nor OMC, BP3 and B-MDM showed clear and dose-dependent antiestrogenic activities at the ERα or to ERβ (Schreurs et al., 2002).

For the few differences between our results obtained in the hERα assay and other *in vitro* and *in vivo* assays, various reasons might be responsible. On one hand, UV filters may be active towards the ERβ, but not the ERα, or may display different activities to both receptors, similar to the methoxychlor metabolite HPTE, which showed selective agonist activity through ERα and antagonistic activity through ERβ (Gaido et al., 1999; Waters et al., 2001). This holds for 4MBC, which binds preferably to the ERβ (Schlumpf et al., 2004). Furthermore, yeasts have only a low capability for metabolism, hence estrogenic UV filter metabolites formed *in vivo* or in metabolically active *in vitro* systems are not identified in the hERα assay. Besides,

estrogenicity may occur via indirect action not governed by the estrogen receptor, and therefore be detectable *in vivo* but not *in vitro*. Differences in species susceptibility may also partly be responsible for distinct estrogenic activities of a compound. Nevertheless the hERα assay can be applied to estimate estrogenicity of UV filters in fathead minnows (Kunz et al., 2006).

hAR agonistic and antagonistic UV filters. Agonistic and antagonistic UV filters were also found in the hAR assay. To our surprise 6 UV filters (BP2, HMS, IMC, OMC, OC, OS) exhibited submaximal to full androgenic activity, and full antiandrogenic activity in addition. On the other hand, 4MBC, 3BC, BP1, 4HB, 4DHB, BP3, BP4, BS, PS, PABA, and OD-PABA acted like pure antiandrogens lacking any androgenic activity.

Our study shows for the first time that at least 6 UV filters exhibit androgenic activity, and an unexpectedly high number of 16 UV filters showed full antiandrogenic activity via binding to the hAR, while Et-PABA was the only UV filter with partial antiandrogenic activity. Our results are consistent with findings on BP3, BP4, OD-PABA, 3BC, and 4MBC in the human breast carcinoma MDA-kb2 reporter gene assay (Ma et al., 2003) (Tab 8). However, HMS and OMC were not active in MDA-kb2 cells, probably due to the low endogenous occurrence of hAR in this cell line. Only one study investigated androgenicity of UV filters in fish, but neither 4MBC nor OMC possessed androgenic activity towards the AR in male medaka (Inui et al., 2003). This may be related to higher metabolic activity *in vivo*, rather than differences between human and fish AR. As androgenic or antiandrogenic activities of UV filters in fish have not yet been evaluated, potential *in vivo* consequences for aquatic organisms remain elusive. Similar to the estrogen receptor, the androgen receptor lacks high substrate specificity, and some estrogenic compounds also activate the androgen receptor (Gaido et al., 1997; Sohoni and Sumpter, 1998). In our study, we only found this to be the case for BP2, which showed strong estrogenic and androgenic activity.

Interestingly, we did not find pure hAR agonists; all 6 androgenic UV filters possessed antiandrogenic activity in addition by inhibiting DHT activity. Wong et al (1995) noted that metabolites of hydroxyflutamide caused AR activation in the absence of the androgen. However in the presence of DHT, these compounds had antagonistic activity. It was suggested that mixed ligand hAR dimers, i.e. agonist (natural androgens) and antagonist, bound in the same dimer, are required for antagonism, whereas the same ligand dimers of sufficiently high affinity promote receptor activation (Wong et al., 1995). This mode of action was for example found for p,p'-DDE (Schrader and Cooke, 2000). The UV filters BP2, HMS, OMC, OS, OC, and IMC possibly followed the same mode of action, showing both partial to full hAR-agonist activity in the absence of DHT, and full hAR antagonism in the presence of DHT.

Another mode of action is proposed for the 11 antiandrogenic UV filters (camphor derivates, benzophenones, salicylates, Et-PABA and OD-PABA) that lacked any androgenic activity. AR

antagonists prevent or reduce the binding of AR to the androgen response elements (ARE), either by preventing - via diverse conformational changes – the formation of the AR dimer, or by preventing the DNA-binding of the dimer (Kelce et al., 1995; Kelce and Wilson, 1997). AR antagonists fail to induce a transcriptionally competent conformation in the AR ligand binding domain *in vitro*, but reverse the agonist-elicited conformational change in the ligand binding domain (Kallio et al., 1994; Kuil and Mulder, 1995). We hypothesize that the purely antagonistic UV filters bind to the hAR, either to the hormone binding site - or similar to the ER - to a second non-hormone binding site of the hAR. Hence, transactivation may have failed, because of conformational changes preventing dimer formation or ARE binding similar to FT and vinclozolin act *in vitro* and *in vivo* (Kelce and Wilson, 1997),and p,p'-DDE (Kelce et al., 1995).

The antiandrogenic UV filters showed unexpectedly high activities coupled with potencies that were between up to 4-fold higher and only 40-fold lower than that of the known antiandrogen flutamide (FT), used in prostate cancer treatment. BP1 was the most potent UV filter, followed by 4HB, PS, BP2, BS, OS, BP3, OD-PABA, 4MBC, 3BC, IMC, 4DHB, OC, HMS, OMC, BP4 and Et-PABA. The UV filters PS, BP2, and 4DHB produced non-monotonic dose response curves, which may result from superimposition of monotonic dose response curves of component biological reactions (Maness et al., 1998; Conolly and Lutz, 2004). The high antiandrogenic activity of UV filters *in vitro*, with EC_{50} values similar or considerably lower than that of FT may have consequences *in vivo* (Vinggaard et al., 2005). Flutamide, for example, leads to significant reduction of androgen-dependent play behavior in neonatal male rats at 50 mg/kg/day (Hotchkiss et al., 2004). Consequently, antiandrogenic UV filter may result in overall estrogenic effects *in vivo*, which will be assessed for aquatic organisms in forthcoming studies in our laboratory.

Multiple hormonal activities of UV filters. This is the first report on multiple hormonal activities for as many as 18 UV filters (Tab. 7). Surprisingly, all UV filters but PABA and PEG25-PABA exhibited multiple hormonal activities *in vitro*. An unexpectedly high number of 10 UV filters displayed as much as three distinct hormonal activities each, having two of them in common, namely being hAR and hERα antagonists. Androgenic activity in combination with hAR and hERα antagonism was found for the alkylated salicylate derivates HMS and OS, as well as the cinnamate derivates IMC, OMC and OC, all being alkyl substituted, which might explain the lack of estrogenic activity (Blair et al., 2000). On the other hand, the salicylates BS and PS, the benzophenones BP3 and BP4, and the camphor derivate 3BC displayed estrogenic besides antiestrogenic and antiandrogenic activities. This combination of hormonal activities is also found for bisphenol A (Lee et al., 2003). BP2 carrying as much as four hydroxy groups displayed three distinct hormonal activities; full estrogenicity and androgenicity besides antiandrogenicity. Hence, BP2 is the only UV filter being a hERα- and hAR agonist, which has

been observed with other estrogenic compounds that also activate the androgen receptor (Gaido et al., 1997; Sohoni and Sumpter, 1998).

Six UV filters exhibited two distinct hormonal activities each. Antiandrogenicity was either coupled with full antiestrogenicity (4MBC and OD-PABA) or with full estrogenicity (BP1, 4DHB, 4HB, Et-PABA). As for 4MBC and OD-PABA, the combination of antiestrogenicity and antiandrogenicity was observed for the antiestrogen 4HT acting as a weak antiandrogen (Sohoni and Sumpter, 1998). Weak estrogenic compounds such as FT and bisphenol A were found to act as strong antiandrogens in addition (Lee et al., 2003), which is also the case for the UV filters BP1, 4DHB, 4HB and Et-PABA. Also a variety of pesticides (Andersen et al., 2002; Kojima et al., 2004), p,p'-DDE (Kelce et al., 1995; Gaido et al., 1997), methoxychlor

and its metabolites (Gaido et al., 2000) exhibit both estrogenic and antiandrogenic activities. It is not yet known, what structural features are necessary for exhibiting these combinations of multiple hormonal activities. Our data indicate that presumably many compounds in the aquatic environment will have complex interactions with the endocrine system that cannot strictly be categorized into one single hormonal activity.

Comparison of in vitro and in vivo estrogenicity of Et-PABA in fish. The estrogenic activity *in vitro* with hERα and rtERα was matched *in vivo* for Et-PABA (Fig. 6C). The *in vitro* activity of Et-PABA towards the rtERα (EC50 3.0 x 10^{-4} M) was slightly higher than to the hERα (8.7 x 10^{-4} M), but the potency of Et-PABA differed considerably, because of the lower sensitivity of rtERα towards E2, making Et-PABA much more potent towards the fish ER. This is consistent with our previous findings (Kunz et al. 2006), where for most of the 23 investigated UV filters, relative sensitivities of rtERα and hERα systems varied within one order of magnitude, indicating that the main difference between the two receptors is their sensitivity towards E2. In juvenile fathead minnows Et-PABA led to VTG induction at 2.7 x 10^{-5} M, which is lower than *in vitro*. Similar results were obtained for the UV filters benzophenone-1 and benzophenones-2, which were also pure estrogens *in vitro* towards the hERα and rtERα, and led to VTG induction in juvenile fathead minnows (Kunz et al. 2006). The underestimation of effects in vitro assays determined in our study by receptor-based in vitro assays as compared to in vivo effects, was previously observed in similar studies, where estrogenicity of municipal wastewater were analysed by the hERα assay and VTG induction in male medaka (Huggett et al., 2003; Sapozhnikova et al., 2005).
When looking at the potencies relative to E2 calculated for Et-PABA, we find that the rtERα assay and in vivo data are in a similar range (38'500- and 43'900-times weaker than E2, respectively), whereas the hERα assay strongly underestimates the potency of Et-PABA (3 million times weaker than E2). This data indicate that the rtERα *in vitro* data are more accurate for prediction of the *in vivo* activity than the hERα. Hence, hormonal activity of UV filters

should be assessed by a suite of species related *in vitro* and *in vivo* assays, confirming the approach previously suggested by Kunz et al. (2006), where we showed that an adequate *in vitro* assay should be able to predict *in vivo* activity of the most potent compounds for further *in vivo* testing. However, ligand-receptor based systems, such as the yeast assays used in this study, have some limitations. For example these systems are not able to detect alterations in the biosynthesis or degradation of a specific hormone and likewise indirect effects on endocrine feedback loops or precursors are not deteted.

Conclusions

Our findings demonstrate that many UV filters occurring in aquatic systems possess multiple hormonal activities *in vitro*, including estrogenicity, antiestrogenicity, androgenicity and antiandrogenicity through interaction with hERα and/or hAR. Although most of the UV filters exert hormonal effects at concentrations that are orders of magnitude higher than in the environment, wide distribution and exposure to UV filter mixtures may have environmental consequences due to additive effects. The UV filters 4MBC, BP3, BP4, OMC, OC and HMS that repeatedly were detected in the aquatic environment, may contribute with their multiple hormonal activities in a complex manner to the mixture of endocrine disrupting chemicals already present in surface water and fish. For most of the UV filters with multiple hormonal activities residues in the aquatic environment and in biota are not yet known, and therefore their environmental relevance remains elusive. The fact that all 18 UV filters and one metabolite showed receptor ligand binding via transactivation - surprisingly most of them multiple bindings - reveals a complex picture of the hormonal activities of UV filters. *In vitro* antiestrogenicity and antiandrogenicity seems even more important than estrogenicity. Moreover, six UV filters have been demonstrated to be androgenic for the first time. Our results support the fact that many estrogenic compounds may have more than one mode of action, and that their diverse hormonal activities are more complex than previously expected. The antiestrogenic, antiandrogenic and androgenic activities of UV filters *in vitro* are of significant scientific and practical interest. Despite the limitations of yeast systems such as a lower metabolic capacity as compared to many animal cell systems and their restriction to ligand-receptor based mechanisms compared to *in vivo* systems which also can detect indirect effects on endocrine feedback loops and precursors, they are an elegant and efficient tool to identify endocrine disrupting compounds that may further be investigated *in vivo*, especially regarding adverse effects in fish.

With Et-PABA, a pure hERα agonistic UV filter, we demonstrated that the hERα assay is a useful tool to predict the *in vivo* estrogenicity in fish, although it tends to underestimate potencies of the estrogenic compounds, whereas the rtERα assay predicts the *in vivo* estrogenicity of Et-PABA in juvenile fathead minnows more accurately. Possible implications of our findings should be addressed by the analysis of UV filters in the aquatic environment. Ongoing studies in

fish in our laboratory will show how the diverse hormonal activities of UV filters will translate into the activity *in vivo*. These findings may be of considerable practical importance due to the frequent use of UV filters in sunscreens, skin care products, cosmetics and in a variety of different materials prone to contaminate aquatic ecosystems.

Acknowledgement

We would like to thank John Sumpter, Brunel University, Uxbridge, U.K., for providing the recombinant hERα and hAR yeast cells, Farzad Pakdel, University of Rennes, for providing the recombinant rtERα, Héctor Galicia, Springborn Smithers Laboratories (Europe) AG, Horn, Friedrich Jüttner and Jakob Pernthaler, University of Zürich, and Daniel Gygax, Chemistry Department (FHBB), for support. This work was funded by the Swiss National Science Foundation (National Research Programme NRP50, contract 4050-066554 to K. Fent).

References

Andersen, H. R., Vinggaard, A. M., Rasmussen, T. H., Gjermandsen, I. M., Bonefeld-Jorgensen, E. C., 2002. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity *in vito*. Toxicol. Appl. Pharmacol. 179, 1-12.

Balmer, M., Buser, H. R., Müller, M. D., Poiger, T., 2005. Occurrence of some organic UV filters in wastewater, in surface waters, and in fish from Swiss lakes. Environ. Sci. Technol. 39, 953-962.

Blair, R. M., Fang, H., Branham, W. S., Hass, B. S., Dial, S. L., Moland, C. L., Tong, W., Shi, L., Perkins, R., Sheehan, D. M., 2000. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: Structural diversity of ligands. Toxicol. Sci.(54), 138-153.

Bowers, J. L., Tyulmenkov, V. V., Jernigan, S. C., Klinge, C. M., 2000. Reservatrol acts as a mixed agonist/ antagonist for estrogen receptors a and b. Endocrinology 141(10), 3657-3667.

Buser, H. R., Balmer, M. E., Schmid, P., Kohler, M., 2006. Occurence of UV filters 4-methylbenzylidene camphor and octocrylene in fish from various swiss rivers with inputs from wastewater treatment plants. Environ. Sci. Technol. 40, 1427-1431.

Collins, B. M., McLachlan, J. A., Arnold, S. F., 1997. The estrogenic and antiestrogenic activities of phytochemicals with the human estrogen receptor expressed in yeast. Steroids 62, 365-372.

Conolly, R. B., Lutz, W. K., 2004. Nonmonotonic dose-response relatinships: mechanistic basis, kinetic modeling, and implications for risk assessment. Toxicol. Sci. 77, 151-157.

Dudley, M. W., Sheeler, C. Q., Wang, H., Khan, S., 2000. Activation of the human estrogen receptor by the antiestrogens ICI 182,780 and tamoxifen in yeast genetic systems: Implications for their mechanism of action. P. Nat. Acad. Sci. USA 97(7), 3696-3701.

Felix, T., Hall, B. J., Brodbelt, J. S., 1998. Determination of benzophenone-3 and metabolites in water and human urine by solid-phase microextraction and quadrupole ion trap GC-MS. Anal. Chim. Acta 371, 195-203.

Gaido, K. W., Leonard, L. S., Lovell, S., Gould, J. C., Babaï, D., Portier, C. J., McDonnel, D. P., 1997. Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. Toxicol. Appl. Pharm. 143, 205-212.

Gaido, K. W., Leonard, L. S., Maness, S. C., Hall, J. M., McDonnel, D. P., Saville, B., Safe, S., 1999. Differential interaction of the methoxychlor metabolite 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane with estrogen receptors a and b. Endocrinology 140(12), 5746-5753.

Gaido, K. W., Maness, S. C., McDonnell, D. P., Dehal, S. S., Kupfer, D., Safe, S., 2000. Interaction of methoxychlor and related compounds with estrogen receptor α and ß, and androgen receptor: Structure-activity studies. Mol. Pharm. 58, 852-858.

Gangloff, M., Ruff, M., Eiler, S., Duclaud, S., Wurtz, J. M., Moras, D., 2001. Crystal sturcture of a mutant hERa ligand-binding domain reveals key structural features for the mechanism of partial agonism. J. Biol. Chem 276(18), 15059-15065.

Garey, J., Wol.ff, M. S., 1998. Estrogenic and antiprogestagenic activities of pyrethroid insecticides. Biochem. Biophys. Res. Com. 251, 855-859.

Hany, J., Nagel, R., 1995. Nachweis von UV-Filtersubstanzen in Muttermilch. Deut. Lebensm.-Rundsch. 91(11), 341-345.

Hedden, A., Müller, V., Jensen, E. V., 1995. A new interpretation of antiestrogen action. Ann. NY Acad. Sci. 761, 109-120.

Holbech, H., Norum, U., Korsgaard, B., Bjerregaard, P., 2002. The chemical UV-filter 3-benzylidene camphor causes an oestrogenic effect in an *in vivo* fish assay. Pharmacol. Toxicol. 91, 204-208.

Hotchkiss, A. K., Ostby, J. S., Vandenbergh, J. G., Gray, L. E., 2004. An environmental antiandrogen, vinclozolin, alters the organization of play behaviour. Physiol. Behav. 79, 151-156.

Inui, M., Adachi, T., Takenaka, S., Inui, H., Nakazawa, M., Ueda, M., Watanabe, H., Mori, C., Iguchi, T., Miyatake, K., 2003. Effect of UV-screens and preservatives on vitellogenin and choriogenin production in male medaka (*Oryzias latipes*). Toxicology 194, 43-50.

Jensen, E. V., Khan, S. A., 2004. A two-site model for antiestrogen action. Mechan. Ageing Dev. 125, 679-682.

Jordan, V. C., Phelps, E., Lindergreen, J. U., 1987. Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res. Treat. 10, 31-35.

Kallio, P. J., Jänne, O. A., Palvimo, J. J., 1994. Agonists, but not antagonists, alter the conformation of the hormone-binding domain of androgen receptor. Endocrinology 134, 998-1001.

Kelce, W. R., Stone, C. R., Laws, S. C., Gray, L. E., Kemppainen, J. A., Wilson, E. M., 1995. Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. Nature 375, 581-585.

Kelce, W. R., Wilson, E. M., 1997. Environmental antiandrogens: developmental effects, molecular mechanisms, and clinical implications. J. Mol. Med. 75, 198-207.

Kenakin, T., 2004. Principles: Receptor theory in pharmakology. Trends Pharmacol. Sci. 25(4), 186-192.

Kojima, H., Katsura, E., Takeuchi, S., Niiyama, K., Kobayashi, K., 2004. Screening for estrogen and androgen receptor activities in 200 pesticides by *in vitro* reporter gene assays using Chinese Hamster ovary cells. Environ. Health Perspect. 112(5), 524-531.

Kuil, C. W., Mulder, E., 1995. Effects of androgens and antiandrogens on the conformation of the androgen receptor. Ann. NY Acad. Sci. 761, 351-354.

Kunz, P. Y., Galicia, H. F., Fent, K., 2004. Assessment of hormonal activity of UV filters in tadpoles of frog *Xenopus laevis* at environmental concentrations. Mar. Environ. Res. 58, 431-435.

Kunz, P. Y., Galicia, H. F., Fent, K., 2006. Comparison of *in vitro* and *in vivo* estrogenic activity of UV filters in fish. Toxicol. Sci. 90, 349-361.

Le Guével, R., Pakdel, F., 2001. Streamlined b-galactosidase assay for analysis of recombinant yeast response to estrogens. BioTechniques 30, 1000-1004.

Lee, H. J., Chattopadhyay, S., Gong, E. Y., Ahn, R. S., Lee, K., 2003. Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. Toxicol. Sci. 75, 40-46.

Ma, R., Cotton, B., Lichtensteiger, W., Schlumpf, M., 2003. UV filters with antagonistic action at androgen receptors in the MDA-kb2 cell transcriptional-activation assay. Toxicol. Sci. 74, 43-50.

Maness, S. C., MacDonnell, D. P., Gaido, K. W., 1998. Inhibition of androgen receptor dependent transcriptional activity by DDT isomers and methoxychlor in HepG2 human hepatoma cells. Toxicol. Appl. Pharmacol. 151, 135-142.

Miller, D., Wheals, B. B., Beresford, N., Sumpter, J. P., 2001. Estrogenic activity of phenolic additives determined by an in vitro yeast bioassay. Environ. Health Perspect. 109, 133-138.

Mueller, S. O., Kling, M., Firzani, P. A., Mecky, A., Duranti, E., Shields-Botella, J., Delansorne, R., Borschard, T., Kramer, P. J., 2003. Activation of estrogen receptor a and ERb by 4-methylbenzylidene-camphor in human an rat cells: comparison with phyto- and xenoestrogens. Toxicol. Lett. 142, 89-101.

Panter, G. H., Hutchinson, T. H., Länge, R., Lye, C. M., Sumpter, J. P., Zerulla, M., Tyler, C. R., 2002. Utility of a juvenile fathead minnow screening assay for detecting (anti-)estrogenic substances. Environ. Toxicol. Chem. 21(2), 319-326.

Parks, L. G., Lambright, C. S., Orlando, E. F., Guillette, L. J. J., Ankley, G. T., Gray, L. E., 2001. Masculinization of female mosquitofish in kraft mill effluent-contaminated fenholloway river water is associated with androgen receptor agonist activity. Toxicol. Sci. 62, 257-267.

Petit, F., Valotaire, Y., Pakdel, F., 1995. Differential functional activities of rainbow trout and human estrogen receptor expressed in the yeast *Sacharomyces cerevisiae*. Eur. J. Biochem. 233, 584-592.

Pike, A. C. W., Brzozowski, A. M., Hubbard, R. E., Bonn, T., Thorsell, A. G., Engström, O., Ljunggren, J.,

Gustafsson, J. A., Carquist, M., 1999. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full agonist. EMBO J. 18(17), 4608-4618.

Poiger, T., Buser, H. R., Balmer, M., Bergqvist, P. A., Müller, M. D., 2004. Occurrence of UV filter compounds from sunscreens in surface waters: regional mass balance in two Swiss lakes. Chemosphere 55, 951-963.

Routledge, E. J., Sumpter, J. P., 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ. Toxicol. Chem. 15(3), 241-248.

Routledge, E. J., Sumpter, J. P., 1997. Structural features of alkylphenolic chemicals associated with estrogenic activity. J. Biol. Chem. 272(6), 3280-3288.

Schlecht, C., Klammer, H., Jarry, H., Wuttke, W., 2004. Effects of estradiol, benzophenone-2 and benzophenone-3 on the expression pattern of the estrogen receptors (ER) alpha and beta, the estrogen receptor-related receptor 1 (ERR1) and the aryl hydrocarbon receptor (AhR) in adult ovariectomized rats. Toxicology 205, 123-130.

Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B., Lichtensteiger, W., 2001. In vitro and in vivo estrogenicity of UV screens. Environ. Health Perspect. 109, 239-244.

Schlumpf, M., Schmid, P., Durrer, S., Conscience, M., Maerkel, K., Henseler, M., Gruetter, M., Herzog, I., Reolon, S., Ceccatelli, R., Faass, O., Stutz, E., Jarry, H., Wuttke, W., Lichtensteiger, W., 2004. Endocrine acitivity and developmental toxicity of cosmetic UV filters - an update. Toxicology 205, 113-122.

Schrader, T. J., Cooke, G. M., 2000. Examination of selected food additives and organochloride food contaminants for androgenic activity *in vitro*. Toxicol. Sci. 53, 278-288.

Schreurs, R. H., Lanser, P., Seinen, W., Van der Burg, B., 2002. Estrogenic activity of UV filters determined by an in vitro reporter gene assay and in vivo transgenic zebrafish assay. Arch. Toxicol. 76, 257-261.

Schreurs, R. H., Legler, J., Artola-Garicano, E., Sinnige, T. L., Lanser, P. H., Seinen, W., Van der Burg, B., 2004. In vitro and in vivo antiestrogenic effects of polycyclic musks in zebrafish. Environ. Sci. Technol. 38(4), 997-1002.

Schultis, T., Metzger, J. W., 2004. Determination of estrogenic activity by LYES-assay (yeast estrogen screen-assay assisted by enzymatic digestion with lyticase). Chemosphere 57(11), 1739-45.

Schultz, T. W., Seward, J. R., Sinks, G. D., 2000. Estrogenicity of benzophenones evaluated with a recombinant yeast assay: Comparison of experimental and rules-based predicted activity. Environ. Toxicol. Chem. 19, 301-304.

Smith, C. L., O'Malley, B. W., 2004. Coregulator function: A key to understanding tissue specificity of selective receptor modulators. Endocr. Rev. 25(1), 45-71.

Sohoni, P., Sumpter, J. P., 1998. Several environmental oestrogens are also anti-androgens. J. Endocrinol. 158(3), 327-339.

Soto, A. M., Justicia, H., Wray, J. W., Sonnenschein, C., 1991. p-Nonyl-phenol: an estrogenic xenobiotic released from „modified" polystyrene. Environ. Health Perspect. 92, 167-73.

Stephenson, R. P., 1997. A modification of receptore theory (Reprinted from Brit J Pharmacol, vol 11, pg 379, 1956). Brit. J. Pharmacol. 120(4), 106-120.

Thomas, K. V., Hurst, M. D., Matthiessen, P., McHugh, M., Smith, A., Waldock, M. J., 2002. An assessment of in vitro androgenic activity and the identification of the environmental androgens in the United Kingdom estuaries. Environ. Toxicol. Chem. 21, 1456-1461.

Tinwell, H., Lefevre, P. A., Moffat, G. J., Burns, A., Odum, J., Spurway, T. D., Orphanides, G., Ashby, J., 2002. Confirmation of the uterothropic activity of 3-(4-methylbenzylidine)camphor in the immature rat. Environ. Health Perspect. 100(5), 533-536.

Tyler, C. R., Jobling, S., Sumpter, J. P., 1998. Endocrine disruption in wildlife: a critical review of the evidence. Crit. Rev. Toxicol. 28(4), 319-61.

Vinggaard, A. M., Jacobsoen, H., Metzdorff, S. B., Andersen, H. R., Nellemann, C., 2005. Antiandrogenic effects in short-term in vivo studies of the fungicide fenarimol. Toxicology 207(1), 21-34.

Waters, K. M., Safe, S., Gaido, K. W., 2001. Differential gene expression in response to methoxychlor and estradiol through ERa, ERb and AR in reproductiv tissues of female mice. Toxicol. Sci. 63, 47-56.

Wong, C., Kelce, W. R., Sar, M., Wilson, E. M., 1995. Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. J. Biol. Chem. 270, 19998-20003.

Yamasaki, K., Takeyoshi, M., Yakabe, Y., Sawaki, M., Takatsuki, M., 2003. Camparison of the reporter gene assay for ER-alpha antagonists with the immature rat uterotrophic assay of 10 chemicals. Toxicol. Lett. 142, 119-131.

Chapter 3

Estrogenic activities of UV filter mixtures

Petra Y. Kunz and Karl Fent

submitted to *Toxicology and Applied Pharmacology*

Abstract

UV-absorbing chemicals (UV filters) are widely used for protection against UV radiation in sunscreens and in a variety of cosmetic products and materials. Depending on the breadth and factor of UV protection, they are added as single compounds or as a combination thereof. Some UV filters have estrogenic activity, but their activity and interactions in mixtures are largely unknown. In this work, we analysed 8 commonly used UV filters, which are pure or partial hERα agonists, for their estrogenic activity in mixtures in a recombinant yeast assay carrying the human estrogen receptor alpha (hERα). Equi-effective mixture combinations of two and four pure hERα agonists alone or in combination with 17-β estradiol (E2) were assessed. Concentration-response relationships for all UV filter combinations were recorded and used to calculate predictions of their joint effects by employing the concentration addition (CA) and independent action (IA) model. Furthermore, mixtures of eight pure and partial hERα agonistic UV filters were studied at concentrations giving an increase of 10% of basal activity, (BC10) and at the no-observed-effect-concentrations (NOEC) of each compound. Most binary mixtures comprising of pure hERα agonists behaved according to the CA concept and showed a synergistic activity at all mixture combinations. Only in combination with benzophenone-1, antagonistic activity was observed at some effect levels. All mixtures of four pure hERα agonists alone or including E2 showed synergistic activity at low effect concentrations (BC10). This occurred even at equi-effective concentrations that were at the NOEC level of each single compound. Mixtures of 8 UV filters alone or with E2 at the BC10 and NOEC level showed synergistic activity. Hence, there were substantial mixture effects even though each UV filter was present at its NOEC level. These results show that significant interactions occur in UV filter mixtures, which is important for the hazard and risk assessments of these personal care products.

Introduction

Sunscreens and cosmetics including lipsticks, skin lotions, hair sprays, hair dyes, shampoos, and numerous other products like moldings, jet ink, tyres and fabrics contain increasing amounts of UV filters, which scatter and reflect UV light. Increasing numbers of materials contain UV filters in order to prevent degeneration by UV radiation. Increased sunlight protection factors are used for preventing negative effects on the human skin. Generally this requires higher percentages of UV filters in the products and a growing use of combinations of different UV filters for absorbing UVA, UVB and UVC light.

UV filters may penetrate the human skin when applied in sunscreens or cosmetics and indeed in human urine, benzophenone-3 (BP3) and its metabolite benzophenone-1 (BP1) have been detected 4 hr after application of commercially available sunscreen products to the skin (Felix et al., 1998). Residues of BP3 and OMC were also detected in human breast milk samples up to 445 ng/g lipid (Hany and Nagel, 1995). Commonly, combinations of several different UV filters are added to sunscreens, cosmetics and other materials, depending on the desired protection factor and range. Thus, mixtures of UV filters with distinct estrogenic activities are applied on human skin and human exposure can occur via dermal absorption or through the food chain, namely fish consumption.

Because of their persistence, UV filters may ultimately reach aquatic systems directly via recreational activities (bathing), and indirectly via wastewater. 4-Methylbenzylidene camphor (4MBC), BP3, octyl methoxycinnamate (OMC), and octocrylen (OC) were detected in lakes and wastewater up to 2'400 ng/L (Balmer et al., 2005; Buser et al., 2006). In southern California, benzophenone and OMC were found in the range of 0.26 to 5.61 μg/L in raw and treated drinking water (Loraine and Pettigrove, 2006). UV filters were also found in fish of up to 2 mg/kg lipid (Balmer et al., 2005). Recently, bioconcentration factors of 230-980 have been determined in fish (Kunz et al., in press). Once UV filters enter the aquatic ecosystems, they join in a mixture of other potential xenoestrogens.

In recent years, it has become evident that some UV filters are estrogenic *in vitro* as demonstrated in MCF-7 cells (Schlumpf et al., 2001), recombinant cell lines (Schreurs et al., 2002; Mueller et al., 2003) and recombinant yeast systems carrying the human ERα (Routledge and Sumpter, 1997; Schultz et al., 2000; Kunz and Fent, submitted). Moreover, a high proportion of commonly used UV filters has been found to exhibit multiple hormonal activities *in vitro* (Kunz et al., in press). Estrogenic activity has also been observed in rats (Durrer *et al.* 2005; Schlumpf *et al.* 2001; Seidlová-Wuttke *et al.* 2004) and fish (Holbech et al., 2002; Inui et al., 2003; Kunz et al., 2006).

As UV filters are applied as compound mixtures, it is important to understand their activity in mixture combinations. Moreover, this is the situation in the environment. Currently, however, the interactions in UV filter mixtures are largely unknown. A previous study using mixtures of estrogenic compounds including 2,4-dihydroxybenzophenone combined at concentrations below the no-observed-effect-concentrations (NOEC) demonstrated significant mixture effects in the yeast hERα assay (Rajapakse et al., 2002; Silva et al., 2002). The effect of mixtures of four estrogenic UV filters on pS2-gene transcription was studied recently in MCF-7 cells. Mixtures of two (BP1, BP3) and four compounds (BP1, BP3, OMC, 4MBC) showed additive activity (Heneweer et al., 2005). Currently, it remains unclear whether and how the estrogenic and/or antiestrogenic activity of single UV filters at low aqueous concentrations contributes to mixture effects. In our study, we focus on these questions as such knowledge is needed for evaluating the potential hazards and risks of estrogenic UV filters for humans and on aquatic life.

The question of mixture activity has gained increasing attention in the last few years (Kortenkamp and Altenburger, 1998). Mixture effects of estrogenic compounds can be calculated based on the activities of individual mixture components. The joint action of weak estrogenic compounds was recently shown to be based on the concept of concentration addition (CA) *in vitro* (Payne et al., 2000; Rajapakse et al., 2002; Silva et al., 2002) and *in vivo* in fish (Brian et al., 2005) In our study, we applied two competing pharmacological concepts for the calculation of expected additive mixture effects. The concept of CA (Loewe and Muischnek, 1926) assumes that components of a mixture act in a similar way, such that one can be replaced by an equal fraction of an equieffective concentration of another, without weakening the overall mixture effect. Synergistic or antagonistic additivity can then be determined by applying the method of isoboles (Loewe and Muischnek, 1926; Kortenkamp and Altenburger, 1998). The concept of independent action (IA) on the other hand assumes that mixture effects are the result of interactions of individual mixture components with different modes of action and mixture components that are present below zero effects are not expected to contribute to the total mixture effect (Bliss, 1939).

In the present work we investigate the activities of 2, 4 and 8 UV filters alone and combined with E2 at different effect levels and at the NOEC. We selected commonly used UV filters, which were demonstrated to be pure hERα agonists or partial hERα agonists *in vitro* (Kunz et al., in press). We addressed the influence of partial agonism for the mixture activity, as little attention is given to this property when estrogenic chemicals are assessed in mixtures. By investigating multi-component mixtures of commonly used pure and partial hERα agonistic UV filters we test the hypothesis that multi-component mixtures comprising of pure hERα agonists follow the model of CA, leading to additive or even synergistic estrogenic activity. In addition, we

hypothesize that multi-component mixtures of pure and partial hERα agonists also follow the CA model, but lead to an antagonistic overall mixture effect, due to the antiestrogenic properties of the partial hERα agonists.

Material and Methods

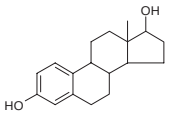
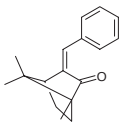
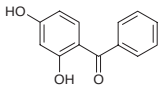
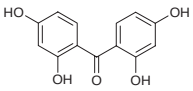
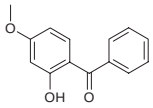
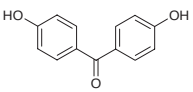
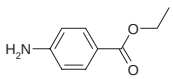
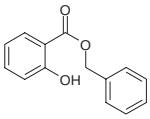
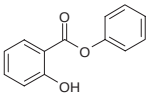
Chemicals. 17β-Estradiol (E2) was purchased from Fluka AG (Buchs, Switzerland). UV filters (Tab. 1) were obtained as follows: Benzophenone-1 (BP1), benzophenone-2 (BP2), benzophenone-3 (BP3), 4,4'-dihydroxybenzophenone (DHB), benzylsalicylate (BS), phenlysalicylate (PS) and ethyl-4-aminobenzoate (Et-PABA) were obtained from Fluka AG (Buchs, Switzerland). 3-Benzylidene-camphor (3BC) was purchased from Induchem (Volketswil, Switzerland). All compounds used were >99% pure. Stock solutions were made in ethanol and stored in the dark at 4°C. Analytical grade ethanol (free of UV filter) was purchased from T.J. Baker (Stehelin AG, Basel, Switzerland). Bidestilled water was produced using a Jencons Autostill double D-ionstill (Renggli AG, Rotkreuz, Switzerland).

Yeast estrogen assay expressing human estrogen receptor alpha (hERα assay). The estrogen-inducible expression system used was kindly gifted by J. Sumpter (Brunel University, U.K.) and is described in detail by (Routledge and Sumpter, 1996). In brief, the yeast (*Saccharomyces cerevisiae*) genome carries a stably integrated DNA sequence of the human estrogen receptor (hERα). Yeast cells also contain expression plasmids carrying estrogen responsive elements (ERE), regulating the expression of the reporter gene lacZ (encoding the enzyme β-galactosidase). Thus, when an active ligand (i.e. 17β-estradiol or an estrogenic UV filter) binds to the receptor, β-galactosidase is synthesised and secreted into the medium, leading to a colour change of chromogenic substrate chlorophenol red ß-D-galactopyranoside (CPRG) from yellow to red.

Preparation of assay media. Preparation of medium compounds was done as previously described by (Routledge and Sumpter, 1996). All components except $Fe_2(SO_4)_3$, inotisol, copper (II) sulphate (Fluka, Buchs, Switzerland) and CPRG (Roche, Basel, Switzerland), were purchased from Sigma (Glattbrugg, Switzerland). Ten-times concentrated stock cultures of both recombinant yeast strains, were stored at -20°C in 0.5 mL aliquots. Every four months (shelf life), both yeast strains were replaced with new -20°C stock cultures. Prior to the experiments the growth medium was inoculated with 125 µL ten times concentrated yeast stock, and incubated at 28°C for 24 h on an orbital shaker. The final assay medium was prepared by seeding 50 mL fresh growth medium with $4x10^7$ yeast cells. Then 0.5 mL CPRG was added.

hERα assay procedure. The yeast assay was carried out within a type II laminar flow. Stock solutions of standards and chemical mixtures were serially diluted in ethanol. Aliquots of 10

Table 1. Chemical structures, molecular weight and CAS numbers of compounds analysed.

Compound	Chemical structure	Molecular Weight (g/L)	CAS
17β-Estradiol (E2)		272.39	50-28-2
3-Benzylidene camphor (3BC)		240.34	15087-24-8
Benzophenone-1 (BP1)		214.22	131-56-6
Benzophenone-2 (BP2)		246.22	131-55-5
Benzophenone-3 (BP3)		228.25	131-57-7
4,4'-Dihydroxybenzophenone (4DHB)		214.22	611-99-4
Ethyl-4-aminobenzoate (Et-PABA)		165.19	94-09-7
Benzyl salicylate (BS)		228.25	118-58-1
Phenyl salicylate (PS)		214.22	118-55-8

Abbrevations: CAS, Chemical Abstracts Service

µL were then transferred 96-well optically flat-bottomed microtitre plates (Greiner Bio-One, Huber AG, Basel, Switzerland) and ethanol was allowed to evaporate to dryness. In all assays experiments were repeated at least twice and each plate contained a positive control with E2 in triplicates. The tested compounds were analysed in quadruplicates. A blank row with ethanol was added in order to control for a possible CPRG conversion due to the medium components or materials alone. After adding aliquots of 200 µL of the final assay medium to the plates, they were sealed with plate sealers (Micronic, Vitaris AG, Baar, Switzerland) and shaken vigorously for 2 min on a titre plate shaker before incubation at 32 °C. After the 72 hours of incubation, plates were shaken vigorously for 2 min and left for 1 h to allow yeasts to settle. The plates were than read at absorbances of 540 nm and 620 nm, using a Tecan GENios plate reader (Tecan AG, Männedorf, Switzerland).

Calculations. The absorbance-measurement at 540 nm (CPRG) and 620 nm (turbidity) allowed for subsequent correction for turbidity (yeast growth), as follows:

$$\text{Corrected absorbance} = \text{chemical absorbance}_{540\,\mathrm{m}} - \left[\begin{array}{l}\text{chemical absorbance}_{620\,\mathrm{m}} - \\ \text{blank absorbance}_{620\,\mathrm{m}}\end{array}\right] - \text{blank absorbance}_{540\,\mathrm{m}}$$

Statistical analysis. For curve fitting and EC50 calculations, the corrected absorbance values versus the logarithm of concentration were plotted, whereby the best fit from a number of non-linear regression models was selected for final data analysis. In this study, we used the four-parameter logistic equation (Hill equation) to fit full dose-response curves according to

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(LogE50 - X)\cdot HillSlope}},$$

where X is the logarithm of concentration and Y is the response showing a sigmoid shape. LogEC50 is the concentration of the mixture yielding half maximal effects and HillSlope is the slope parameter. Coefficient of determination (R^2), residuals and 95% confidence intervals were calculated so as to verify that the fitted curve represents the data correctly. The runs test was carried out in order to ensure that the model chosen to fit the curve does not significantly deviate from the data. Submaximal dose-response curves were fitted using the best fit from a number of non-linear regression models. Curve fitting was carried out using GraphPad Prism software (GraphPad Software Inc., San Diego, USA).

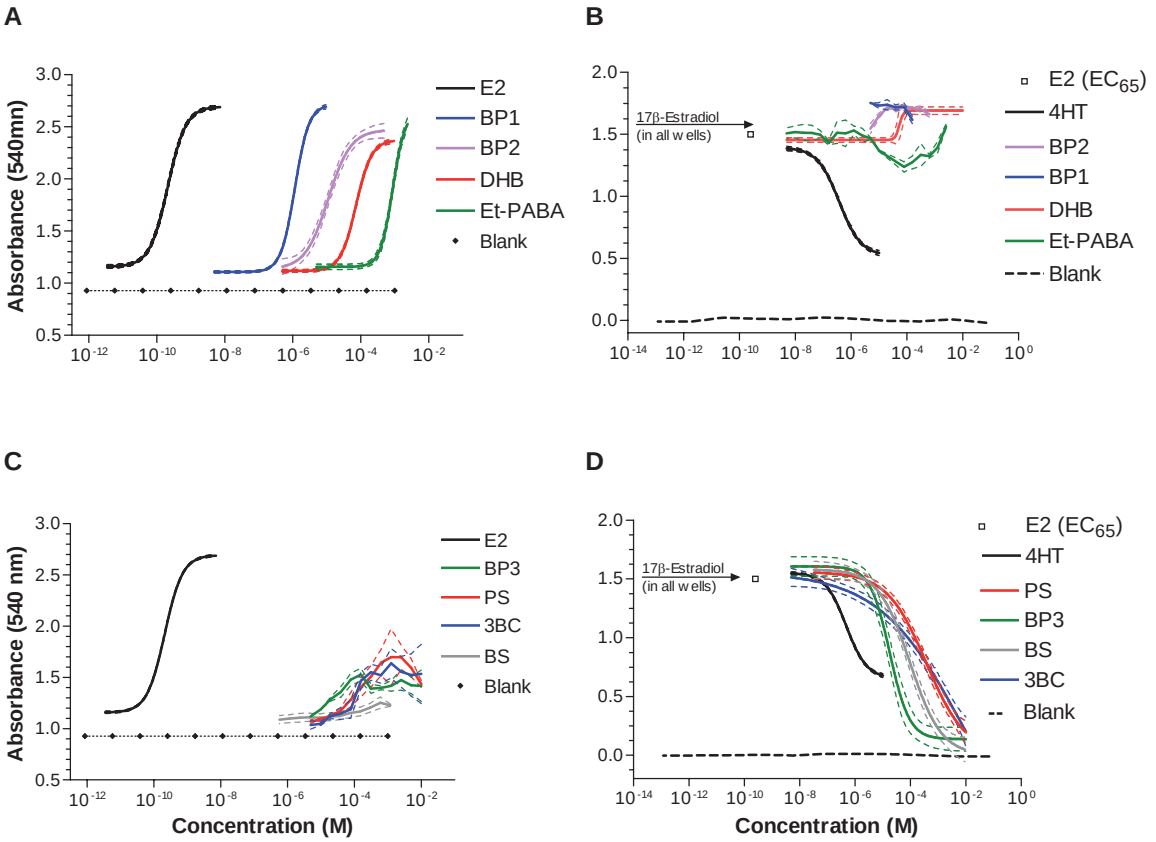


Figure 1 Estrogenic and antiestrogenic activities of single compounds in the hERα assay. (A) Estrogenic activity of pure hERα agonists. (B) Antiestrogenic activity of pure hERα agonists. (C) Estrogenic activity of partial hERα agonist. (D) Antiestrogenic activity of partial hERα agonists. Data shown are means ± 95% CI band (3 experiments with 4 replicates each). Data from Kunz and Fent, in press.

Efficacies, i.e. cure heights of full and submaximal dose response curves were calculated as follows for single compounds and mixtures:

$$Efficacy_{UVfilter} = \left[\frac{1}{Top_{Standart} - Bottom_{Standart}}\right] \cdot Top_{UVfilter} - Bottom_{UVfilter} \quad ,$$

where the curve height ($Top_{UV\ filter} - Bottom_{UV\ filter}$) of the compound was compared with the curve height of the corresponding standard ($Top_{Standard} - Bottom_{Standard,}$ efficacy set at 100 %). Estrogenic activities were calculated for dose-response curves of single compounds and mixtures by normalizing the data (blank or basal absorbance=1) and using non-linear regression analysis to determine the concentration at which each compound caused elicits 50% of its maximal activity (EC50). Subsequently relative potencies (RP), i.e. estrogenic activities were derived for each compound and mixture by dividing the EC50 for E2 by the EC50 values of each single compound or mixture (Table 2).

Mixture design and testing. The compounds used for the mixture experiments were previously found to be either pure or partial hERα agonists in the hERα assay (Kunz et al., 2006; Kunz et al., in press). BP1, BP2, DHB and Et-PABA were full hERα agonists exhibiting full dose-

Table 2. Estrogenic effects of 8 UV filters and E2 in the yeast hERα assay and their concentrations in isoeffective mixtures of two, four and eight compounds.

	Efficacy[a]	RP[b]	Asymmetric hill function parameters			Binary mixure ratios (M)			Multiple mixture ratios (M)	
		1/...	Hill Slope	Max[c]	EC50 (M)	EC25	EC50	EC75	BC10	NOEC*
E2	100%	1	1.632± 0.38	1.536± 0.16	2.59± 1.19E-10	1.08E-10	2.11E-10	4.14E-10	3.91E-11	6.32E-12
BP1	96%	5'000	1.300	1.604	1.15E-06	4.96E-07	1.15E-06	2.69E-06	1.93E-07	1.68E-08
BP2	91%	21'000	2.107	1.336	1.09E-05	6.47E-06	1.09E-05	1.85E-05	3.55E-06	6.16E-07
4DHB	91%	170'000	1.902	1.256	7.34E-05	4.12E-05	7.34E-05	1.29E-04	2.39E-05	3.28E-06
Et-PABA	87%	3'500'000	2.395	1.490	8.96E-04	5.49E-04	8.69E-04	1.37E-03	2.82E-04	6.39E-05
3BC	21%	1'300'000	1.000	0.326	3.10E-04	--	--		1.75E-03	1.56E-06
BP3	18%	45'000	2.322	0.324	1.86E-05	--	--		2.49E-05	1.28E-06
BS	12%	860'000	1.000	0.157	1.66E-04	--	--		5.05E-03	8.38E-07
PS	32%	480'000	1.699	0.529	1.10E-04	--	--		6.89E-05	3.69E-06

Abbreviations: For abbreviations of listed compounds see Table 1; EC75, EC50 and EC25, concentration of the compound exhibiting 75%, 50% and 25%, respectively, of its total effect; BC10, concentration of the compound exhibiting a 10% increase of the baseline absorbance, used instead of EC10 because of too large differences in curve steepness and height; NOEC, calculated as a 0.3% increase of the baseline absorbance. EC-, BC- and NOEC-values of compounds derived from 3 experiments with four replicates each. --, not calculated. Values for E2-standard are given in mean S.E.M. (n= 9). Value of compounds from 3 experiments with four replicates each.

[a] Efficacy, effect (curve height) of a compound given as percentage of the effect of E2.

[b] RP, relative potency gives ratio of the EC50 of a compound divided by the EC50 of E2.

[c] Max, curve-height = top – bottom.

response curves (Fig. 1A) with no antiestrogenic activity (Fig. 1B). BP3, BS, PS and 3BC are partial hERα agonists with submaximal dose-response curves (Fig. 1C). They show full antiestrogenic activity when co-exposed with E2 (Fig. 1D)(Kunz et al., in press). Table 2 gives data about their dose-response curves and relative potencies (RP), which served as a basis for our mixture experiments.

In our experiments the compounds were mixed at iso-effective concentrations; this means that the single compounds would lead to the same effect when tested alone. Mixture experiments with combinations of two and four compounds with or without E2 were conducted with pure hERα agonistic UV filters only (Fig. 1 A-B). In order to investigate mixture activities of these UV filters, binary mixture experiments were conducted first by mixing effect concentrations of the single UV filters for the effect levels of EC25, EC50 and EC75, respectively. Experiments with mixtures of four pure hERα agonists and mixtures of 8 UV filters (+/- E2), comprising of pure and partial hERα agonists (Fig. 1 A and C), were then conducted at lower mixture levels in order to investigate activities at low concentrations (10% effect level, BC10) and below the NOEC (NOEC).

In order to get equipotent multi-component mixtures of 4 or 8 UV filters, with and without E2, we used non-linear regression analysis for the normalized dose-response curves of each mixture component to determine the concentration at which each compound caused a 10 % (BC10) increase of basal hERα activity. The NOEC was defined as an increase of 0.3 % of basal hERα activity. We used equi-effective concentrations based on absorbance and baseline instead of EC-mixture levels for the multi-compound mixtures, because thereby we bypassed differences in curve height and slope. We used this approach to calculate NOECs, because they generally denote the highest concentration at and below which the response of exposed organisms do not depart significantly from untreated controls. They are calculated by the Dunnet's statistical test. Hence, the NOEC may not represent a true "zero-effect", but the effect may not be detected in the assay, because it is too weak. In order to prevent testing weak undetectable effects instead of NOEC we used the EC*x* point estimates, which are discussed to replace NOEC in risk assessment (Van der Hoeven, 1997).

Calculations of predicted mixture effects. The joint effects of a mixture with known composition were calculated on the basis of the Hill regression models for each UV filter in the mixture by using the CA concept, the toxic unit approach, and independent action (IA).

In the CA model (Loewe and Muischnek, 1926), compounds act similarly, on the same receptor site and can replace each other. Each compound in the mixture contributes individually to the mixture-effect in proportion to its concentration. Therefore the mixture ratio (the relative abundance of the compound in the mixture) has to be known and dose-response data for each

individual compound has to be available that produces the same effect as the mixture on its own. Thus assuming that the combined effect of the mixture with *n* components is concentration additive, the following equation will hold for any effect level:

$$\sum_{i=1}^{n} \frac{c_i}{EC_i} = 1 \tag{1}$$

where c_i denotes the concentration of the compound *i* in a mixture yielding to an effect *E* and EC_i the concentration of *i* needed to produce the same effect on its own.

The concentration c_i of the compound *i* in the mixture is related to the total mixture concentration by:

$$c_i = p_i \cdot EC_{mix} \tag{2}$$

where the ratio p_i, is the concentration of i relative to the total mixture concentration EC_{mix} producing the total mixture effect *E*. Substitution of c_i in equation (1) gives:

$$\sum_{i=1}^{n} \frac{p_i \cdot EC_{mix}}{EC_i} = 1 \tag{3}$$

and rearranging yields

$$EC_{mix} = \left[\frac{\sum_{i=1}^{n} p_i}{EC_i} \right]^{-1} \tag{4}$$

The concentrations EC_i where calculated from the concentration-response curves of the single compounds by using the inverse expression of the asymmetric Hill function.

Mixtures that are found to follow the concept of CA were investigated for additivity, synergism or antagonism by using the method of isoboles (Loewe and Muischnek, 1926). For binary mixtures it is carried out by constructing graphs that show curves describing combinations of two compounds A and B, which produce the same specific effect (isobole). The axes of the graph are the doses of the two compounds on a linear scale. A line is drawn between the iso-effective doses A and B, representing additivity of the mixture. For mixtures with more than three components the toxic unit approach (Berenbaum, 1985) can be used. Additivity (zero interaction) of a mixture is expressed if equation (1) equals 1. Each fraction (c_i/EC_i) of equation (1) represents the concentration of a mixture component scaled for its relative toxicity and is generally referred to as the toxic unit of that component (Altenburger et al., 2000). If a given mixture deviates from 1, a synergistic mixture effect is indicated by values < 1, whereas an

antagonistic mixture effect result in values > 1. This method is suitable to analyze combinations of compounds, irrespective of the shape of their individual dose-response curves and can also be used in the case of partial agonism (Kortenkamp and Altenburger, 1998).

In the concept of IA (Bliss, 1939), it is assumed that compounds act on different subsystems and show different modes of action. This leads to effects in mixtures, which are based on individual compound-effects that are greater than zero. This model allows predicted effects of mixtures of known composition to be calculated using the expression:

$$1-\prod_{i=1}^{n}\left[1-E(c_i)\right] \tag{5}$$

where $E(c_i)$ is the effect E produced by the compound i at concentration c. Inherent in this expression is the fact that $E(c_i)$ cannot exceed 1, i.e. $E(c_i)$ is a fraction of a maximal possible effect, making IA a probabilistic model. Therefore when applying this model to our assay effects $AE(c_i)$, a maximal effect E_{max} has to be defined. We used in our assays E2, which gave the highest absorbance value as a reference. The effect of the mixture is expressed relative to the maximal effect of E2.

$$E(c_i)=\frac{AE(c_i)}{E_{\max}} \tag{6}$$

If the concentration-response relationships of all mixture compounds i are described by an appropriate regression model F_i (asymmetric Hill function), the assay effect $AE(c_i)$ can be estimated from the mean effect $F_i(c_i)$, predicted by the regression model. Thus,

$$E(c_i)=\frac{F_i(c_i)}{E_{\max}} \qquad \text{and} \qquad AE(c_i)=F_i(c_i) \tag{7}$$

substitution of $E(c_i)$ in this equations yields

$$c_{mix}=1-\prod_{i=1}^{n}\left[1-\frac{F_i(c_i)}{E_{\max}}\right] \tag{8}$$

In order to assure comparability of the IA predictions with those of CA, the fractional effect in above equation were rescaled by multiplications with E_{max}. Thus

$$E_{mix}=E_{\max}\left[1-\prod_{i=1}^{n}\left[1-\frac{F_i(c_i)}{E_{\max}}\right]\right] \qquad \text{and} \qquad E_{mix}=E_{\max}\cdot e_{mix} \tag{9}$$

Results

Binary mixtures of pure hERα agonists. In all mixture experiments, each UV filters was used at non-cytotoxic concentrations only. The hERα agonistic UV filters (BP1, BP2, DHB and Et-PABA) were first assessed in binary mixtures at three different effect levels each (EC25, EC50 and EC75). The dose-response curves are shown in Figure 2. The estimates calculated for all binary mixtures produced almost identical curves for the concept of CA and IA (not shown in Fig. 2 for clarity reasons). An example of estimated CA and IA curves in binary mixtures is shown in Figure 3. The dose-response curves for the observed and expected mixture effects agreed well, when the mixture was close to additivity and displayed weak antagonism (Fig. 3A), whereas when the mixture was strongly synergistic, the observed curve was shifted considerably to lower concentrations (Fig. 3B).

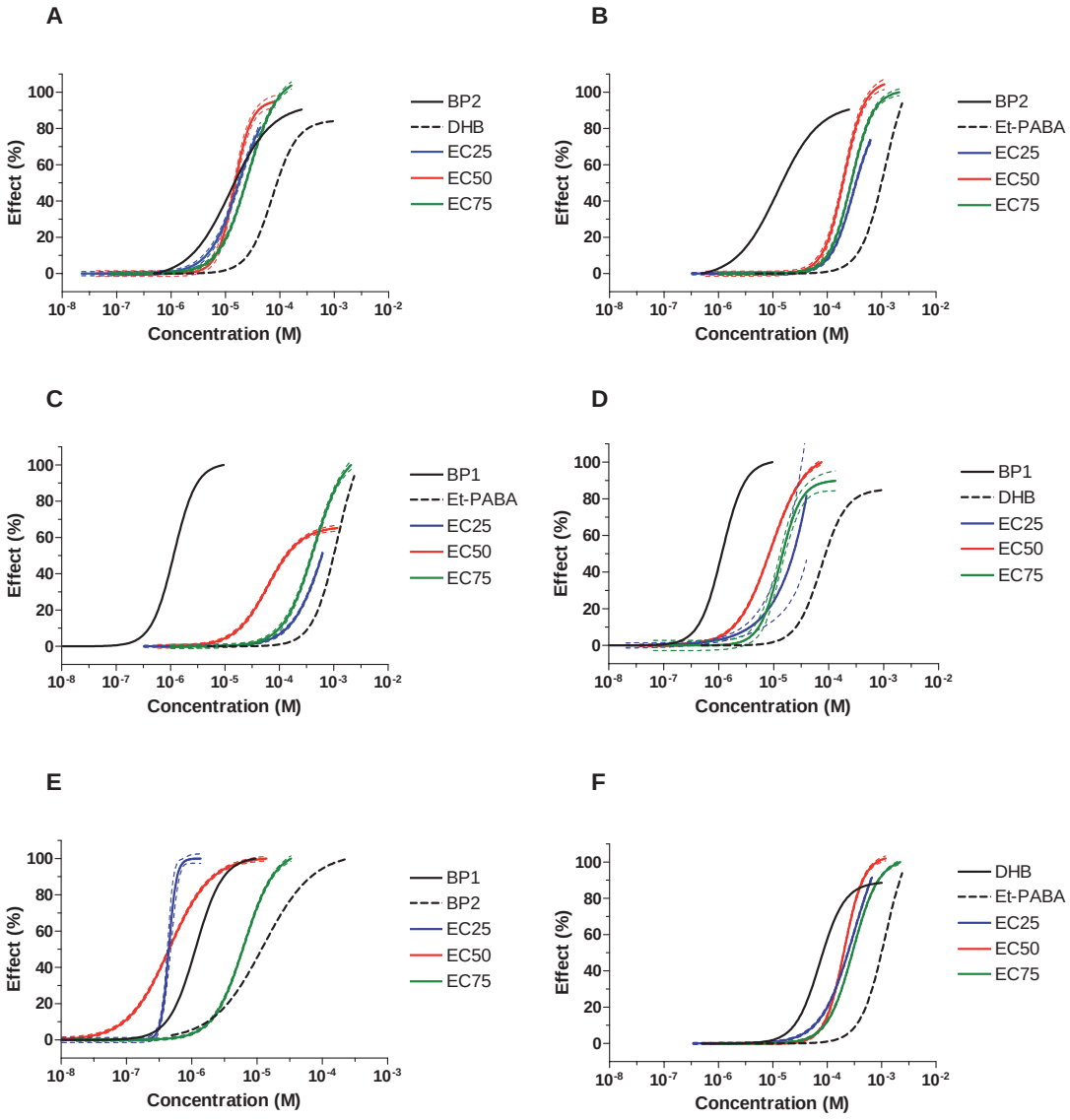


Figure 2 Binary mixtures of pure hERα agonistic UV filters, dose-response curves for mixtures at effect levels EC25, EC50 and EC75. Dotted lines represent dose-response curve of single mixture compounds (data from Kunz and Fent, in press). Results are presented in panels A-F for clarity reasons. Equieffective mixtures of BP2 and DHB (A), BP2 and Et-PABA (B), BP1 and Et-PABA (C), BP1 and DHB (D), BP1 and BP2 (E) and DHB and Et-PABA (F). with CA and IA, isoboles for effect levels.

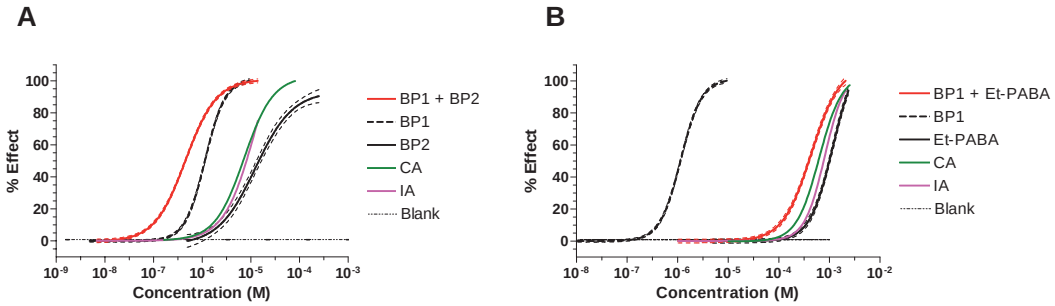


Figure 3 Two examples for estimated CA and IA curves for binary mixtures. (A) Binary mixture of BP1 and Et-PABA at EC75 with weakly antagonistic mixture activity. (B) Binary mixture of BP1 and BP2 at EC50 with synergistic mixture activity. Dotted lines represent dose-response curve of single mixture compounds (data from Kunz and Fent, in press).

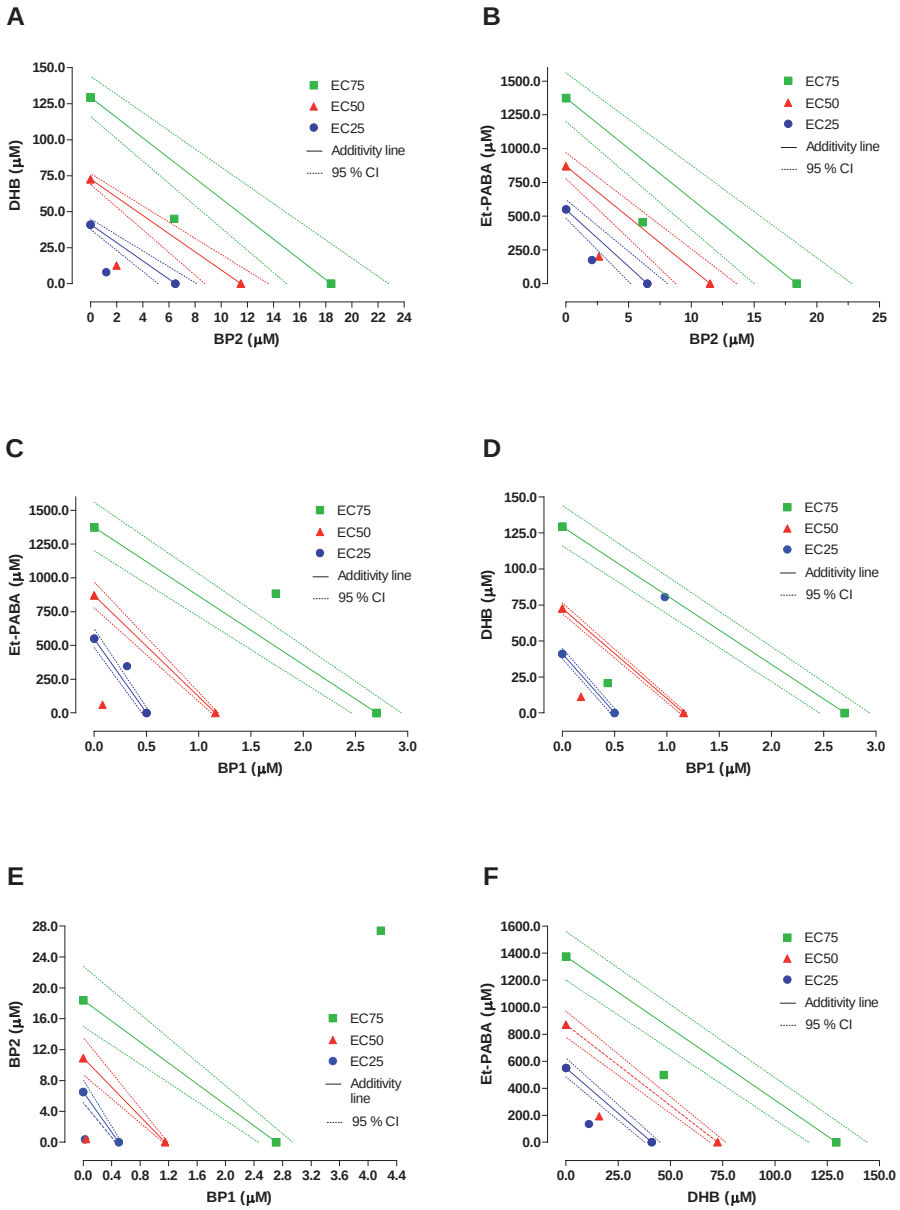


Figure 4 Equi-effective isobole graphs for binary mixtures of pure hERα agonistic UV filters, at effect levels EC25, EC50 and EC75. Dotted lines represent the additivity line with the 95% confidence intervals (CI) band for the single compounds. Results are presented in panels A-F for clarity reasons. Equieffective mixtures of BP2 and DHB (A), BP2 and Et-PABA (B), BP1 and Et-PABA (C), BP1 and DHB (D), BP1 and BP2 (E) and DHB and Et-PABA (F).

In order to evaluate binary mixtures for additivity, synergism or antagonism, we have plotted the results using the isobole method and the results are shown in Fig. 4. Moreover, toxic units were calculated for all mixtures and effect levels (Fig. 5). Binary mixtures with BP1 exhibited antagonism at EC25 (mixture with DHB, Fig. 4A, Fig. 5) or at EC75 (mixture with BP2, Fig. 3B, Fig. 5), or at both mixture levels (Fig. 4C, Fig. 5). However, these were exceptions. For all other binary mixtures and effect levels, we observed synergistic interactions (Fig. 4 D-F, Fig. 5). Generally, lower mixture levels elicited even stronger synergism. For example, the EC25 level of BP2 with DHB and BP1, and DHB with Et-PABA (Fig. 2 A, E-F and Fig. 4 B,D-E) exhibited marked synergism.

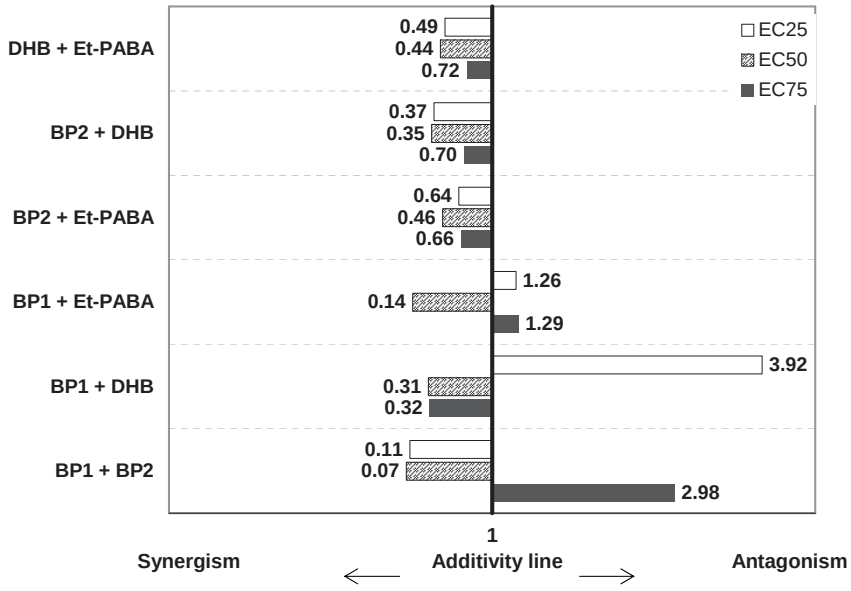


Figure 5 Toxic unit calculations for binary mixtures of pure hERα agonistic UV filters, at effect levels EC25, EC50 and EC75. If the calculated toxic unit equals 1, additivity is assumed; if it is > 1, antagonism is assumed and if it is < 1, synergism.

Mixtures of four UV filters and E2. All mixture of 4 pure hERα agonists (BP1, BP2, DHB and Et-PABA) followed the CA model (Fig. 6) and displayed synergistic activity (Figs. 6, 7). Comparison of their relative potencies with those of single UV filters revealed that mixtures of pure hERα agonists at BC10 and NOEC level led to enhanced relative potencies. Mixture activities reached values similar to the EC50 value of BP1, the most estrogenic UV filter (Fig. 7A).

To our surprise the mixture of four pure hERα agonists (BP1, BP2, DHB and Et-PABA) that were added at concentrations below their NOEC showed a full dose-response curve (Fig. 6C). It exhibited a stronger relative potency (Fig. 7) than the mixture of these 4 UV filters that were mixed at the effect level of BC10. This mixture produced a dose-response curve of lesser height (Fig. 6B) and displayed a lower relative potency than E2 (Fig. 7A). In the mixture of

UV filteradded at their NOEC, the addition of E2 enhanced the relative potency. In case of the mixture at the BC10 level, addition of E2 led to a reduced relative potency (Fig. 7).

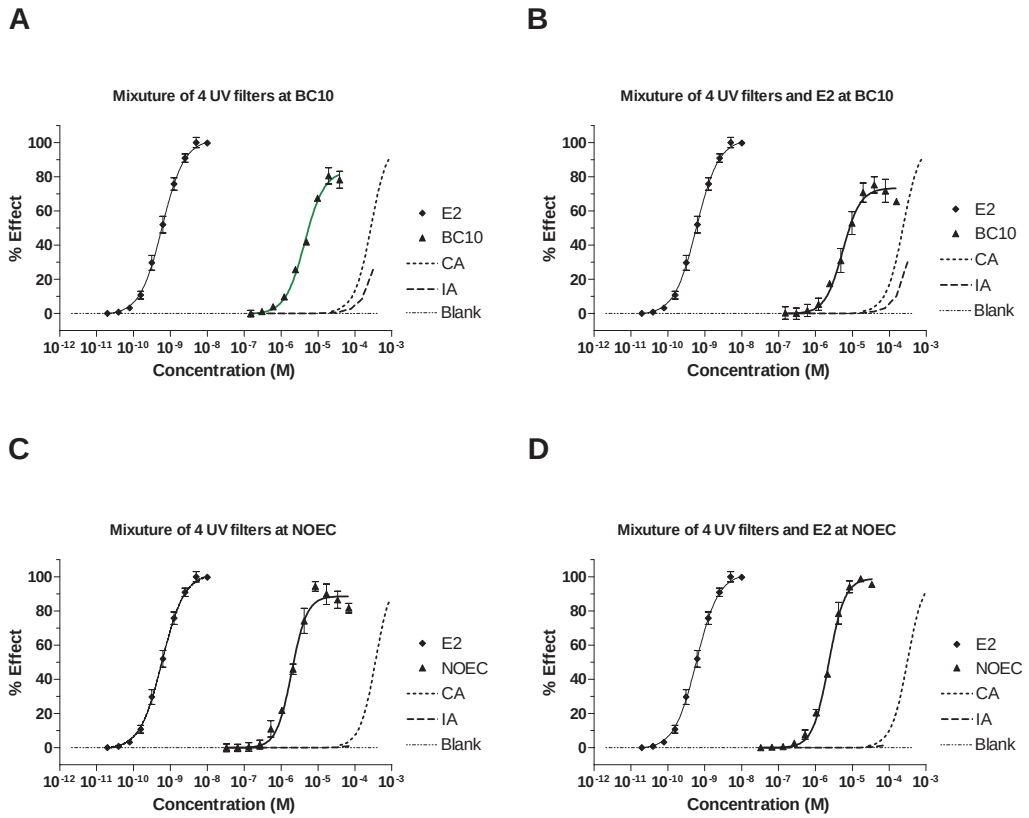


Figure 6 Multi-component mixtures of four pure hEPα agonistic UV filters (BP1, BP2, DHB, Et-PABA), with/without E2, at effect levels BC10 and NOEC. Dotted lines represent estimates for CA and IA curves. Results are presented in panels A-C for clarity reasons. Data shown are means ± S.E.M. (3 experiments with 4 replicates each).

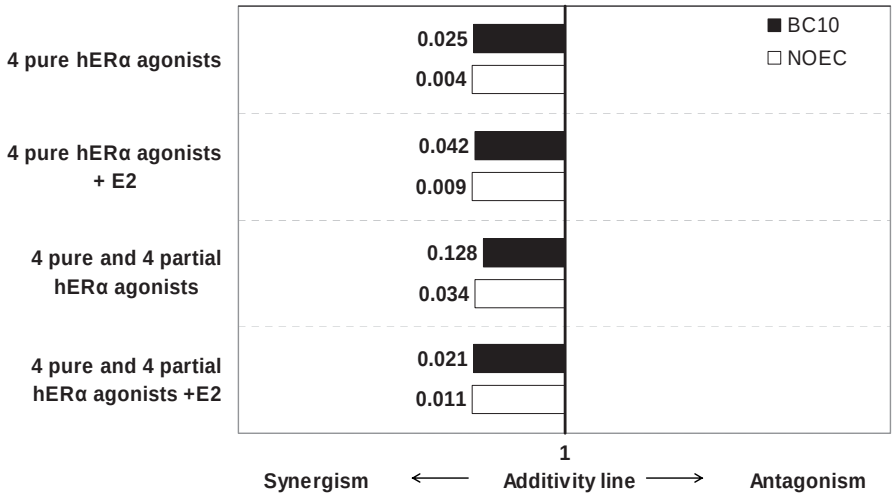


Figure 7 Toxic unit calculations for mixtures of 4 agonistic (BP1, BP2, DHB, Et-PABA), or 4 agonistic and 4 antagonistic (BP3, 3BC, BS, PS) UV filters, with/without E2, at effect levels BC10 and NOEC. If the calculated toxic unit equals 1, additivity is assumed; if it is > 1, antagonism is assumed and if it is < 1, synergism.

Mixtures of eight UV filters and E2. The combination of eight UV filters (BP1, BP2, DHB, Et-PABA, BP3, BS, PS and 3BC) mixed at their NOEC, displayed submaximal dose-response curves that were higher than those of the same compounds mixed at their BC10 (Fig. 8A). The activities of mixtures consisting of 4 pure (BP1, BP2, DHB and Et-PABA) and 4 partial (BP3, BS, PS and 3BC) hERα agonists, alone or in combination with E2, differed from the CA and IA model because of their considerable synergistic activity (Fig. 9). This was the case for both the mixtures at the BC10 and NOEC level. As with binary mixtures, the calculated estimates of the mixtures of 8 UV filters produced very similar curves for the CA and IA model.

Table 3 Parameters of UV filter mixtures in the yeast hERα assay.

	Effect level	Efficacy	RP 1/...	Hill function parameters			Highest mixture concentration (M)
				Hill	Max	EC50 (M)	
BP1 + BP2	EC25	99%	4'389	7.448	1.624	4.456E-07	6.999E.06
	EC50	88%	1'629	1.369	1.044	4.552E-07	1.266E.05
	EC75	100%	2'256	1.000	1.862	1.046E-05	2.110E-05
BP1 + 4DHB	EC25	86%	2'588'699	1.000	1.481	2.445E-04	4.154E-05
	EC50	120%	18'840	1.000	1.478	2.372E-05	1.137E-05
	EC75	90%	68'690	2.302	1.636	1.321E-05	1.320E-04
BP1 + Et-PABA	EC25	52%	4'094'450	1.392	0.904	7.621E-04	5.505E-04
	EC50	66%	132'538	1.441	0.983	6.102E-05	8.713E-04
	EC75	104%	2'548'848	1.552	1.853	4.370E-04	1.378E-03
BP2 + Et-PABA	EC25	76%	1'420'000	2.082	1.349	3.017E-04	5.565E-04
	EC50	115%	1'062'711	2.305	1.912	2.042E-04	8.816E-04
	EC75	107%	1'462'580	2.159	1.852	2.777E-04	1.394E-03
BP2 + DHB	EC25	100%	93'595	1.585	1.800	1.758E-05	4.753E-05
	EC50	96%	97'862	2.727	1.667	1.456E-05	8.406E-05
	EC75	110%	128'339	1.627	1.937	2.624E-05	1.477E-04
DHB + Et-PABA	EC25	94%	2'110'570	1.434	1.617	3.119E-04	5.910E-04
	EC50	96%	1'003'004	2.439	1.901	2.070E-04	9.427E-05
	EC75	103%	2'035'410	1.830	1.961	2.990E-04	1.505E-03
4-Mixture	BC10	72%	7'172	1.64	1.360	4.361E-06	3.093E-04
	NOEC	88%	6'083	1.53	1.360	3.060E-06	6.769E-05
4-Mixture + E2	BC10	83%	7'161	1.82	1.217	5.438E-06	3.093E-04
	NOEC	100%	5'106	2.01	1.828	2.287E-06	6.769E-05
8-Mixture	BC10	41%	181'421	0.83	0.629	1.544E-04	7.199E-03
	NOEC	69%	9'163	1.87	1.092	7.399E-06	7.507E-05
8-Mixture + E2	BC10	78%	244'241	0.48	1.343	1.459E-04	7.199E-03
	NOEC	67%	8'373	1.38	1.178	5.086E-06	7.507E-05

For abbreviations see Table 1 and 2;. value of compounds from 3 experiments with four replicates each.

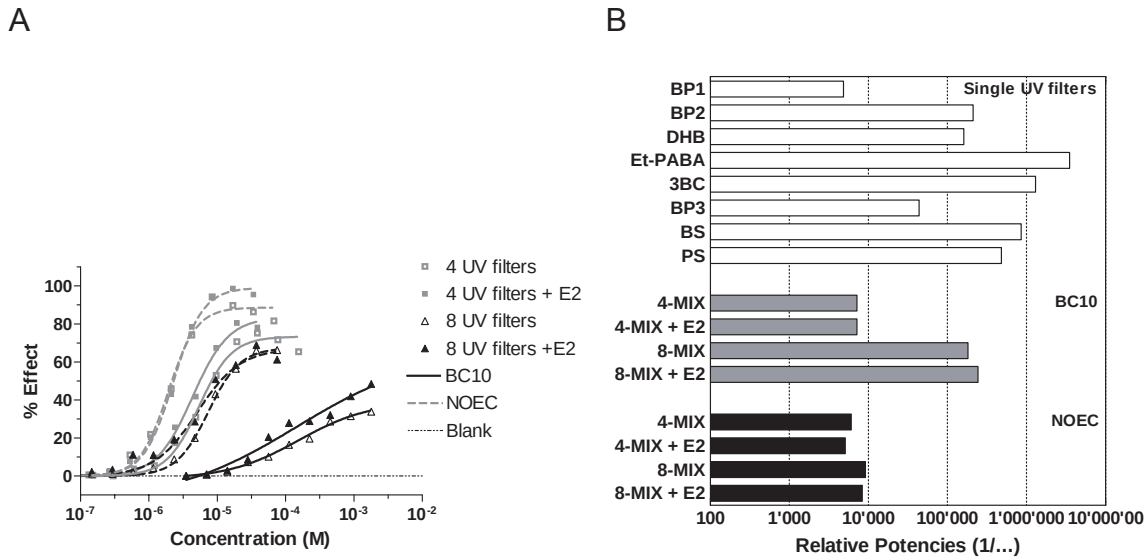


Figure 8 Mixtures of 4 agonistic (BP1, BP2, DHB, Et-PABA), or 4 agonistic and 4 antagonistic (BP3, 3BC, BS, PS) UV filters, with/without E2, at effect levels BC10 and NOEC. (A) Dose-response curves of multi-component at BC10 (solid lines) and NOEC (dotted lines). (B) Calculated relative potencies (RP) compared to E2 for all single compounds, binary mixtures and multicomponent mixtures. Data derive from 3 experiments with 4 replicates each).

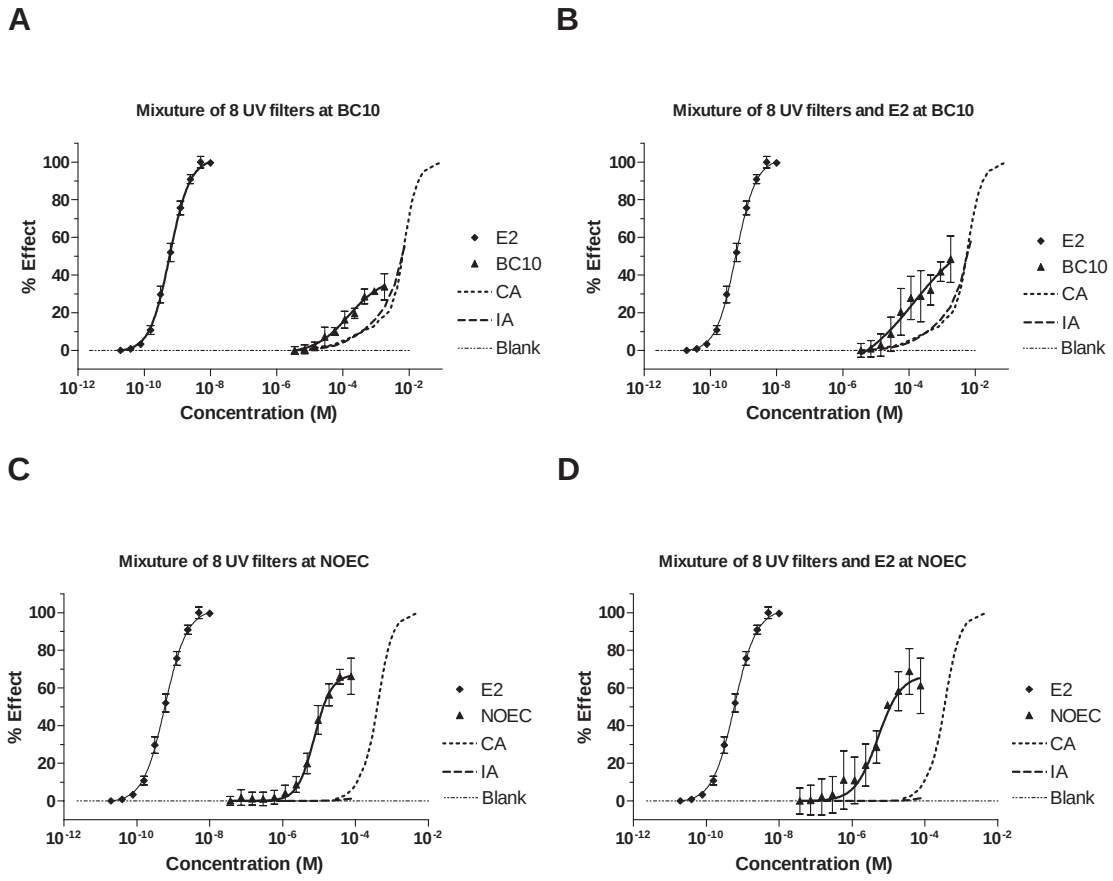


Figure 9 Multi-component of 8 UV filters (BP1, BP2, DHB, Et-PABA, BP3, 3BC, BS PS), with/without E2, at effect levels BC10 and NOEC. Dotted lines represent estimates for CA and IA curves. Results are presented in panels A-C for clarity reasons. Data shown are means ± S.E.M. (3 experiments with 4 replicates each).

Surprisingly, the combined activity of these eight UV filters was synergistic (Figs. 7, 9). When they were mixed with E2 at their NOEC, they showed relative potencies that were two orders of magnitude higher compared to the mixture at the effect level BC10 (Tab. 3, Fig. 7). It is important to note that the relative potencies of the NOEC mixtures were again in the same range as the most estrogenic UV filter found in our study (Fig. 7).

Discussion

UV filters are commonly applied as mixtures in cosmetics and consequently, humans and the aquatic environment are exposed to mixtures of UV filters. In order to better understand the combined mixture activity, we analysed 8 commonly used UV filters with either pure or partial hERα agonistic activity for their estrogenicity. To our surprise the multi-component mixture experiments revealed pronounced synergistic activities of these UV filters when mixed at low effect concentrations. This marked effect was observed for mixtures with pure and partial hERα agonists, even when the individual UV filter concentrations alone were at their NOEC.

Concentration addition concept. The hypothesis that binary and multi-component mixtures of commonly used UV filters would follow the model of CA was supported by our data, although a distinction between the CA and IA model was difficult or even impossible. The estimates for all binary and multi-component mixtures calculated on the assumption of additive interaction produced almost identical curves for CA and IA. This was already observed for mixtures with the same mode of action (hERα agonists) using the same assay (Payne et al., 2000) and for mixtures of compounds with different modes of action in the E-screen (Payne et al., 2001). As the hERα assay is based on the compound's interactions with hERα and does not recognize any other effects, the CA model should produce valid calculations of additive effects. Indeed mixtures of 4 and 8 environmental chemicals in the hERα assay corresponded well with the predictions of CA (Payne et al., 2000; Silva et al., 2002). For our findings we assumed CA because of the reasons mentioned above, and used the isobole method and the toxic unit approach in order to analyse synergistic or antagonistic interactions. The results of these calculations indicate that the binary and multi-component mixtures incorporating pure as well as partial hERα agonists in fact follow the model of CA.

Binary mixtures with pure hERα agonists. In a first series of experiments we mixed different combinations of two UV filters that are pure hERα agonists at three different effect levels. All these binary combinations displayed synergistic interactions. The only exceptions were combinations of UV filters with BP1 at some effect levels. A possible reason for this difference may be related to the large differences in relative potencies of the UV filters; BP1 possesses highest relative potency of all investigated UV filters. Its relative potency is also considerably

higher than that of the other three pure antagonists. Other reasons are deviations from the equi-effectivity of the mixture ratios, because the curve slopes at low and high effect levels differ. When BP1 was combined with BP2 that shows a 4-times lower potency, antagonism was found at the effect level EC75, possibly due to system saturation. This may occur because of the high mixture effect level and/or because of differing slopes. Antagonism also occurred when BP1 was mixed with 4DHB (34-times lower potency) at EC25, or Et-PABA (700-times lower potency) at EC25 and EC75. As a consequence we calculated the equi-effective concentrations of single UV filters for mixtures of 4 and 8 compounds on the basis of equi-effective absorbance values, instead of EC values. Thereby, difficulties arising from differences in curve slope and height were excluded.

Quaternary mixtures with pure hERα agonistic UV filters. Combinations of four compounds mixed at their BC10 and NOEC showed higher activities than predicted by the CA model. The toxic unit approach (Kortenkamp and Altenburger, 1998) demonstrates synergistic interactions for all mixtures at all effect concentrations. The mixture of BP1, BP2, 4DHB and Et-PABA at the NOEC level led to a full dose-response curve and to higher relative potency, as when mixed at BC10 level. This suggests that synergism increases with decreasing mixture levels. Similar to our findings, Silva et al. (2002) found substantial mixture effects and high efficacies when they mixed 8 xenoestrogens at low-dose levels.

The dose-response curves of BC10 mixtures displayed a lower efficacy of 72 to 83%, which seems to be caused by cytotoxicity to the yeasts. Toxicity occurred in the BC10 mixture at concentrations greater than 2×10^{-3} M, whereas the concentrations at which the individual compounds were added to the mixture were far from being toxic on their own. The cytotoxic concentrations of UV filters BP-2 ($>2.5 \times 10^{-3}$ M) and Et-PABA ($>2.5 \times 10^{-3}$ M) were well above those having maximal estrogenicity. This indicates that the cytotoxicity of some UV filters might also be additive or even synergistic in multi-component mixtures, whereas in binary mixtures the interaction of these compounds apparently does not lead to toxicity. The addition of E2 enhanced the efficacy of the mixture, but a stronger relative potency was only observed at the NOEC level. Similarly, a mixture of 11 xenoestrogens combined at their EC01 levels (zero effect level) with E2 led to a shift of the resulting dose-response curve to lower concentrations (Rajapakse et al., 2002).

Multi-component mixtures of pure and partial hERα agonistic UV filters. The strong synergism in mixtures incorporating pure and partial hERα agonistic UV filters was surprising. Synergistic activity was observed, when 4 pure hERα agonists were co-exposed with 4 partial hERα agonists. Due to the antiestrogenic activity of partial hERα agonists an overall antagonistic mixture activity was expected, based on the fact that partial agonists elicit only submaximal

dose-response curves on their own and block the effect of full agonist, when co-exposed with this compound (Stephenson, 1956). Partial agonists may only induce a suboptimal conformational change of the hERα (Pike et al., 1999; Bowers et al., 2000; Gangloff et al., 2001), or bind to a secondary binding site (Dudley et al., 2000; Jensen and Khan, 2004), both of which can lead to steric or ionic interferences resulting in reduced efficacy. The only indication of a weak inhibitory action mediated by the partial agonists was found when 8 UV filters were mixed at their BC10 level. In this case, a considerably lower relative potency was observed compared to a mixture of 4 UV filters (7'161 vs. 181'421).

Surprisingly, the activity of mixtures of 8 UV filters (with or without E2) combined at their NOEC was much more potent than its counterpart at the BC10 level. It showed an enhanced activity, which is highly synergistic and only slightly less potent than the NOEC mixtures with 4 pure hERα agonistic UV filters. Nevertheless, the efficacies were only submaximal, with a slightly decreasing effect at the highest concentration, indicating slight toxicity even at NOEC mixture level. The high estrogenicity and efficacy of the NOEC mixture of 8 UV filters may be explained as follows. At very low doses components with higher affinity to the hERα may bind first compared to components having lower affinity, whilst these may rather act as co-activators. Thereby, partial agonism of a compound seems to have only a minor effect at NOEC mixtures. It was shown that a partial agonist exposed alone or in the presence of low concentrations of a full agonist, the resulting effect is submaximal agonistic or additive, respectively (Stephenson, 1956; Kenakin, 2004).

Possible explanations for the high incidence of synergism found in the yeast hERα assay. Why do low-dose mixtures or mixtures at NOEC result in such marked effects? Low-dose effects of endocrine disrupting chemicals are mediated by endocrine signaling pathways that evolved to act as powerful amplifiers, with the result that large changes in cell function can occur in response to extremely low concentrations (Welshons et al., 2003). Thus, in a mixture some compounds may directly mimic E2, whereas others interfere with the production of metabolism or transport of E2 (Weltje et al. 2005), or with regard to our study, they may interfere with β-galactosidase production in the yeast hERα assay. In multi-compound mixtures at very low doses, mixture components with higher affinity to the ER may first bind to it, whilst other compounds rather act as co-activators. Thereby the tiniest amount of a chemical added could have an effect, because its activity adds to a chemical, which is already there, resulting in no threshold effects (Sheehan et al. 1999). Similar to our findings with UV filters, xenoestrogens at levels below individual non-significant concentrations may indeed enhance estrogenic effects in yeasts and MCF-7 cells (Payne et al., 2000; Rajapakse et al., 2002; Silva et al., 2002), but also in transfection systems (Le Page et al., 2006). Also a binary mixture of E2 and ethinylestradiol was more potent than either of the individual compounds *in vivo* in fish (Thorpe et al., 2003).

However, synergistic interactions of xenoestrogen mixtures have caused particular concern, originating in a study that reported strong synergism of a mixture of weak xenoestrogens (Arnold et al., 1996), which could not be reproduced by other laboratories (Ashby et al., 1997; Gaido et al., 1997; Ramamoorthy et al., 1997). This led to the withdrawal of the original paper (McLachlan, 1997). Recently, Kortenkamp and Altenburger (1998) reinvestigated mixture studies with the isobole method and found that at some effect levels (Gaido et al., 1997; Shekhar et al., 1997) and mixture ratios (Ashby et al., 1997) synergism was overlooked. We used the method of isoboles and the toxic unit calculations in order to evaluate mixtures for additivity, synergism or antagonism. The additional calculations of CA and IA, as well as of relative mixture potencies further support our findings with the isobole method for synergistic interactions.

Thus we hypothesize that the observed synergism in most of the mixtures with UV filters - even when combined at individual NOEC's, and even when partial agonists were added - is unlikely due to the characteristics of the yeast transactivation assay, but rather caused by properties of the UV filters, which may activate and co-activate the hERα and other co-activators and factors via diverse actions. In addition, other signal transduction pathways responsible for estrogen-related effects may also play a role. Hormone receptor agonists are not only determined by ligand-receptor binding, but they may also interact with other factors such as co-regulators that are able to modulate the transcriptional activity of steroid hormone receptors (Katzenellenbogen et al., 1996). In contrast to our observations with UV filters, additive interactions were observed in the same hERα assay for mixtures consisting of other xenoestrogens at their NOEC (Rajapakse et al., 2002; Silva et al., 2002). Significant effects in multi-component mixtures were also found with estrogenic pharmaceuticals (Fent et al., in press), and the interaction was synergistic in some cases. UV filters actually seem to have synergistic activities in mixtures, at least in the hERα assay. However, synergism may also be related to and caused by interactions with co-factors in the yeast system. Thus individual compounds in a mixture may have different activities that may influence transactivation, binding, co-activation and expression of β-galactosidase. In order to prove the synergistic interactions of UV filters in mixtures as observed in our study, further analysis in other systems such as cell-lines are necessary.

Conclusions and possible environmental consequences

Recent studies demonstrate that UV filters possess endocrine disrupting properties at rather high concentrations (Schlumpf et al., 2001; Holbech et al., 2002; Inui et al., 2003; Kunz et al., 2006). Our findings on the pronounced synergistic effects of multi-component mixtures of UV filters combined at their individual NOEC indicate that low UV filter concentrations present on the human skin and in the environment may produce relevant estrogenic activity on their

own, or lead to enhanced estrogenic activities of other xenoestrogens or E2, depending on the mixture components.

Indeed, concentrations of single UV filters in the NOEC mixtures were mostly in the µg/L range, when eliciting highest estrogenic activities. These effect concentrations are close to residual concentrations of UV filters found in the environment. In drinking water and reclaimed wastewater in southern California two UV filters were found in the range of 0.26 to 5.61 µg/L (Loraine and Pettigrove, 2006). Residues of BP3 and OMC were found in human breast milk samples up to 445 µg/kg lipid (Hany and Nagel, 1995) and in fish 4 to 6 different UV filters were identified in the low mg/kg range . Our findings suggest that partial agonistic UV filters do not reduce the overall mixture activity due to antagonism. This seem not to be the case at very low concentrations, were these compound also seem to add to the overall mixture effect.

Our study reveals a novel and more detailed picture of the hormonal activity of UV filter mixtures *in vitro* and discloses unexpected synergistic properties of these compounds. In order to assess, whether our *in vitro* findings will translate into other *in vitro* systems *and in vivo*, further studies are needed. The consequences of mixture activities of UV filters found in this study are of significant scientific and practical interest. For an adequate risk assessment it seems unavoidable to consider compound mixtures, when investigating endocrine disrupting properties of UV filters towards humans and aquatic organisms. Ongoing *in vivo* studies in our laboratory will show, whether *in vitro* mixture activities of UV filters will translate into the activity in fish at environmentally relevant concentrations.

Acknowledgement

We would like to thank John Sumpter, Brunel University, Uxbridge, U.K., for kindly providing the recombinant hERα yeast cells, Friedrich Jüttner and Jakob Pernthaler, University of Zürich, and Daniel Gygax, Chemistry Department (FHNW), for support. This work was supported by the Swiss National Science Foundation (NFP50, contract 4050-066554 to K. Fent).

References

Altenburger, R., Backhaus, T., Boedeker, W., Faust, M., Scholze, M., Grimme, L. H., 2000. Predictability of the toxicity of multiple chemical mixtures in Vibrio fischeri mixtures composed of similar acting chemicals. Environ. Toxicol. Chem. 19, 2341-2347.

Arnold, S. F., Robinson, M. K., Notides, A. C., Guillette, L. J., McLachlan, J. A., 1996. A yeast estrogen screen for examining the relative exposure of cells to natural and xenoestrogens. Environmental Health Perspectives 104(5), 544-548.

Ashby, J., Lefevre, P. A., Odum, J., Harris, C. A., Routledge, E. J., Sumpter, J. P., 1997. Synergy between synthetic oestrogens? Nature 385, 494.

Balmer, M., Buser, H. R., Müller, M. D., Poiger, T., 2005. Occurrence of some organic UV filters in wastewater, in surface waters, and in fish from Swiss lakes. Environ. Sci. Technol. 39, 953-962.

Berenbaum, M. C., 1985. What is synergism? Pharmacol. Rev. 41, 93-141.

Bliss, C. I., 1939. The toxicity of poisons applied jointly. Ann. Appl. Biol. 26, 585-615.

Bowers, J. L., Tyulmenkov, V. V., S.C., J., Klinge, C. M., 2000. Reservatrol acts as a mixed agonist/antagonist for estrogen receptors a and b. Endocrinology 141(10), 3657-3667.

Brian, J. V., Harris, C. A., Scholze, M., Backhaus, T., Booy, P., Lamoree, M., Pojana, G., Jonkers, N., Runnalls, T., Bonfà, A., Marcomini, A., Sumpter, J. P., 2005. Accurate prediction of the response of freshwater fish to a mixture estrogenic chemicals. Environ. Health Persp. 113(6), 721-728.

Buser, H. R., Balmer, M. E., Schmid, P., Kohler, M., 2006. Occurence of UV filters 4-methylbenzylidene camphor and octocrylene in fish from various swiss rivers with inputs from wastewater treatment plants. Environ. Sci. Technol. 40, 1427-1431.

Dudley, M. W., Sheeler, C. Q., Wang, H., Khan, S., 2000. Activation of the human estrogen receptor by the antiestrogens ICI 182,780 and tamoxifen in yeast genetic systems: Implications for their mechanism of action. P. Nat. Acad. Sci. USA 97(7), 3696-3701.

Felix, T., Hall, B. J., Brodbelt, J. S., 1998. Determination of benzophenone-3 and metabolites in water and human urine by solid-phase microextraction and quadrupole ion trap GC-MS. Anal. Chim. Acta 371, 195-203.

Fent, K., Escher, C., Caminada, D.,. Estrogenic activity of pharmaceuticals and pharmaceutical misxtures in a yeast reporter gene system. In press.

Gaido, K. W., Leonard, L. S., Lovell, S., Gould, J. C., Babaï, D., Portier, C. J., McDonnel, D. P., 1997. Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. Toxicol. Appl. Pharm. 143, 205-212.

Gangloff, M., Ruff, M., Eiler, S., Duclaud, S., Wurtz, J. M., Moras, D., 2001. Crystal Structure of a mutant hERa ligand-binding domain reveals key structural features for the mechanism of partial agonism. J. Biol. Chem. 276(18), 15059-15065.

Hany, J., Nagel, R., 1995. Nachweis von UV-Filtersubstanzen in Muttermilch. Deut. Lebensm.-Rundsch. 91(11), 341-345.

Heneweer, M., Musse, M., Van den Berg, J., Sanderson, T., 2005. Additive estrogenic effects of mixtures of frequently used UV filters on pS2-gene transcription in MCF-7 cells. Toxicol. Appl. Pharmaconl.

Holbech, H., Norum, U., Korsgaard, B., Bjerregaard, P., 2002. The chemical UV-filter 3-benzylidene camphor causes an oestrogenic effect in an *in vivo* fish assay. Pharmacol. Toxicol. 91, 204-208.

Inui, M., Adachi, T., Takenaka, S., Inui, H., Nakazawa, M., Ueda, M., Watanabe, H., Mori, C., Iguchi, T., Miyatake, K., 2003. Effect of UV-screens and preservatives on vitellogenin and choriogenin production in male medaka (*Oryzias latipes*). Toxicology 194, 43-50.

Jensen, E. V., Khan, S. A., 2004. A two-site model for antiestrogen action. Mechan. Ageing. Dev. 125, 679-682.

Katzenellenbogen, J. A., O'Mallay, B. W., katzenellenbogen, B. S., 1996. Tripartite steroid hormone receptor pharmacology: Interaction with multiple effector sites as a basis for the cell- and promoterö-specific action of these hormones. Mol. Endocrin. 10, 119-131.

Kenakin, T., 2004. Principles: Receptor theory in pharmacology. Trends Pharmacol. Sci. 2004, 186-192.

Kortenkamp, A., Altenburger, R., 1998. Synergisms with mixtures of xenoestrogens: A reevaluation using the method of isoboles. Sci. Total Environ. 221, 59-73.

Kunz, P. Y., Fent, K., submitted. Multiple hormonal activities of UV filters in vitro.

Kunz, P. Y., Galicia, H. F., Fent, K., 2006. Comparison of *in vitro* and *in vivo* estrogenic activity of UV filters in fish. Toxicol. Sci. 90, 349-361.

Le Page, Y., Scholze, M., Kah, O., Pakdel, F., 2006. Assessment of xenoestrogens using three distinct estrogen receptors and the zebrafish brain aromatase gene in a highly responsive glial cell system. Environ. Health Persp. 114(5), 752-758.

Loewe, S., Muischnek, H., 1926. Über Kombinationswirkungen. Arch Exp Pathol Phramakol 114, 313-326.

Loraine, G. A., Pettigrove, M. E., 2006. Seasonal variations in concentrations of pharmaceuticals and personal care products in drinking water and reclaimed wastewater in southern california. Environ. Sci. Technol. 40, 687-695.

McLachlan, J. A., 1997. Synergistic effect of environmental estrogens: Report withdrawn. Science 277, 462-463.

Mueller, S. O., Kling, M., Firzani, P. A., Mecky, A., Duranti, E., Shields-Botella, J., Delansorne, R., Borschard, T., Kramer, P. J., 2003. Activation of estrogen receptor a and ERb by 4-methylbenzylidene-camphor in human an rat cells: comparison with phyto- and xenoestrogens. Toxicol. Lett. 142, 89-101.

Payne, J., Rajapakse, N., Wilkins, M., Kortenkamp, A., 2000. Prediction and assessment of effects of mixtures of

four xenoestrogens. Environ. Health Persp. 108(10), 983-987.
Payne, J., Scholze, M., Kortenkamp, A., 2001. Mixtures of four organochlorines enhance human breast cancer cell proliferation. Environ. Health Persp. 109(4), 391-397.
Pike, A. C. W., Brzozowski, A. M., Hubbart, R. E., Bonn, T., Thorsell, A. G., Engström, O., Ljunggren, J., Gustafsson, J. A., Carquist, M., 1999. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full agonist. The EMBO Journal 18(17), 4608-4618.
Rajapakse, N., Silva, E., Kortenkamp, A., 2002. Combining xenoestrogens at levels below individual no-observed effect concentrations dramatically enhances steroid hormone action. Environ. Health Persp. 110(9), 917-921.
Ramamoorthy, K., Vyhlidal, C., Wang, F., Chen, I.-C., Safe, S., McDonnell, D. P., Leonard, L. S., Gaido, K. W., 1997. Additive Estrogenic Activities of a Binary Mixture of 2',4',6'-Trichloro- and 2',3',4',5'-Tetrachloro-4-biphenylol. Toxicol. Appl. Pharmacology 147(1), 93-100.
Routledge, E. J., Sumpter, J. P., 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ. Toxicol. Chem. 15(3), 241-248.
Routledge, E. J., Sumpter, J. P., 1997. Structural features of alkylphenolic chemicals associated with estrogenic activity. J. Biol. Chem. 272(6), 3280-3288.
Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B., Lichtensteiger, W., 2001. In vitro and in vivo estrogenicity of UV screens. Environ. Health Persp. 109, 239-244.
Schreurs, R. H., Lanser, P., Seinen, W., Van der Burg, B., 2002. Estrogenic activity of UV filters determined by an in vitro reporter gene assay and in vivo transgenic zebrafish assay. Arch. Toxicol. 76, 257-261.
Schultz, T. W., Seward, J. R., Sinks, G. D., 2000. Estrogenicity of benzophenones evaluated with a recombinant yeast assay: Comparison of experimental and rules-based predicted activity. Environ. Toxicol. Chem. 19, 301-304.
Shekhar, P. V. M., Werdell, J., Basrur, V. S., 1997. Environmental estrogen stimulation of growth and estrogen receptor function in preneoplastic and cancerous human breast cell lines. J. Natl. Cancer Inst. 89, 1774-1782.
Silva, E., Rajapakse, N., Kortenkamp, A., 2002. Something from "nothing" - eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environ. Sci. Tech. 36(8), 1751-1756.
Stephenson, R. P., 1956. Modification of receptor theory. Brit. J. Pharmacol. Chemoth. 11, 379-393.
Thorpe, K. L., Cummings, R. I., Hutchinson, T. H., Scholze, M., Brighty, G., Sumpter, J. P., Tyler, C. R., 2003. Relative potencies and combination effects of steroidal estrogens in fish. Environ. Sci. Technol. 15(37), 1142-1149.
Van der Hoeven, N., 1997. How to measure no effect, part III: Statistical aspects of NOEC and ECx estimates. Environmetrics 8, 241-248.
Welshons, W. V., Thayer, K. A., Judy, B. M., Taylor, J. A., Curran, E. M., Vom Saal, F. S., 2003. Large effects from small exposures. I. Mechanisms for endocrine disrupting chemicals with estrogenic activity. Environ. Health Persp. 111(8), 994-1006.

Chapter 4

Determination of nine organic UV-absorbing compounds in water by gas chromatography-mass spectrometry and high-performance liquid chromatography

Abstract

Ultraviolet-absorbing organic chemicals (UV filters) used in sunscreens, cosmetics and a number of materials for light protection enter surface water directly or via wastewater. Residues of some UV filters have been detected in surface water, wastewater, sediment, and biological samples. Due to widespread use of different UV filters and possible environmental contamination, there is a need for appropriate analytical methods for water samples. Here the development of an analytical technique for determination of 9 UV-filters in water is described. It is based on solid phase extraction combined with either gas chromatography-mass spectrometry for 4-methylbenzylidene camphor and 3-benzylidene camphor, or high-performance liquid chromatography for benzophenone-1, benzophenone-2, benzophenone-3, benzophenone-4, 4,4'-dihydroxy-benzophenone, octyl-methoxycinnamate, 4-methylbenzylidene camphor, 3-benzylidene camphor, and ethyl 4-aminobenzoate. By using three different SPE cartridges and two solvents, recoveries were between 87 and 107% even though the analytes show large differences in polarity. Quantification limits in the µg/L range were achieved with HPLC and/or GC/MS, respectively. By an additional concentration of the water sample during SPE separation by a factor of 250 to 2'500, the quantification limits could be reduced to the low ng/L. The developed HPLC-method allows fast and reproducible determination of a series of UV filters in water samples.

Introduction

Organic ultraviolet-absorbing chemicals (UV filters) are widely used in sunscreens, cosmetics, and for light protection of materials. Various combinations of different UV filters are applied to achieve the required degree of protection. While showering, bathing, and swimming, sunscreens and cosmetics like shampoos are easily washed off and thereby, UV filters may contaminate the aquatic environment directly or via household wastewater and sewage works. Since an increasing number of products contain UV filters, production volumes of these compounds are rising. Residues of some UV filters have been found in surface water, wastewater, sewage sludge and fish (Nagtegaal et al., 1997; Poiger et al., 2001; Balmer et al., 2005; Buser et al., 2006; Loraine and Pettigrove, 2006). Critical chemical properties such as persistence, high lipophilicity and exhibition of estrogenic activity in fish and rats (Schlumpf et al., 2001; Holbech et al., 2002; Inui et al., 2003; Schlumpf et al., 2004; Kunz et al., 2006) make UV filters of potential environmental concern.

Hitherto, UV filters have been analyzed mainly in products such as sunscreens and cosmetics, but also in water, and biological samples such as fish and human breast milk (Balmer et al., 2005; Buser et al., 2006) by means of several methods. In commercial formulations UV filters are quantified by reversed-phase liquid chromatography with UV detection, in order to ensure compliance with the proposed maximum permissible concentrations (Gagliardi et al., 1989; DiNunzio and Gadde, 1990; Felix et al., 1998; Rastogi and Jensen, 1998; Vanquerp et al., 1999; Hauri et al., 2003; Salvador et al., 2005). Gas chromatography with mass spectrometry (GC/MS) was used to determine residues of 5 different UV filters in natural waters (Poiger et al., 2004; Loraine and Pettigrove, 2006), 3 different UV filters in sewage sludge (Plagellat et al., 2006), and 6 different UV filters in fish ranging from 2-125 ng/L, 110-5510 µg/kg, and 26-3’100 µg/kg, respectively. Benzophenone-3 (BP-3) and its metabolites have been measured in water and human urine by SPME and ion trap GC/MS (Felix et al., 1998), as well as by liquid chromatography (LC) in sewage sludge (Plagellat et al., 2006), in rat biological fluids and tissues (Abdel-Nabi et al., 1992; Soeberg et al., 2006).

Generally, UV filters are analyzed either by GC/MS (Chisvert et al., 2001; Chisvert and Salvador, 2002; Balmer et al., 2004; Balmer et al., 2005) when being volatile, lipophilic and non-polar (e.g. 4-MBC, OMC, octocrylene and homosalate) or slightly polar (e.g. BP-3, 4-isopropyldibenzoylmethane and 4-tert.-butyl-4’-methoxy-dibenzoylmethane). HPLC is applied for BP-4, BP-5, OD-PABA (Rastogi and Jensen, 1998; Chisvert et al., 2001; Chisvert and Salvador, 2002; Giokas et al., 2004; Salvador et al., 2005) for water soluble and polar comounds with rather low volatility and/or high molecular weight. This leads to a fairly high instrumental effort, when residues of several UV filters with different chemical characteristics should be detected in experimental waters and in natural waters in particular.

Accurate detection of UV filters in water is crucial for the determination of exposure concentrations in ecotoxicological experiments with aquatic organisms and environmental samples and ultimately, for environmental risk assessment. In particular, a fast and reliable method is needed for the analysis of actual concentrations of UV filters in exposure experiments with aquatic organisms (Kunz et al., 2004; Kunz et al., 2006). For this purpose, we have modified an existing GC/MS method (Poiger et al., 2004; Balmer et al., 2005) for the analysis of two UV filters (4-MBC and 3-BC) in exposure experiments with frogs. We subsequently extended it for the analysis to 9 different UV filters currently in use having different polarity and water solubility by developing a method based on solid phase extraction followed by analysis with HPLC-DAD.

Material and Methods

Reagents and solutions

Benzophenone-1 (BP-1), benzophenone-3 (BP-3), benzophenone-4 (BP-4), ethyl 4-aminobenzoate (Et-PABA), and 4,4'-dihydroxybenzophenone (4DHB) were purchased from Fluka AG (Buchs, Switzerland). Octyl-methoxycinnamate (OMC), 4-methylbenzylidene camphor (4-MBC) and 3-benzylidene camphor (3-BC) were purchased from Merck (Dietikon, Switzerland). Benzophenone-2 (BP-2) was purchased from Aldrich (Fluka AG, Buchs, Switzerland). Benzyl cinnamate was obtained from Fluka AG (Buchs, Switzerland). Analytical grade ethanol (EtOH), methanol (MeOH), dichloromethane ($MeCl_2$) and acetonitrile (ACN) were purchased from T.J. Baker (Stehelin AG, Basel, Switzerland). All compounds used had a purity grade ≥ 99 %.

2-Hexanol and formic acid (FA, puriss, p.a. ~98%) were purchased from Fluka AG (Buchs, Switzerland). Bi-distilled water was produced using a Jencons Autostill double D-ionstill distillator (Renggli AG, Rotkreuz, Switzerland) and additionally filtered through membrane filters (RC 55, 0.45µm, Ø 50 mm, Schleicher & Schuell, Geneva, Switzerland) when used as a HPLC solvent.

Stock standard solutions of the UV filters were freshly prepared in ethanol and stored in the dark at 4°C. Their structures and relevant properties are given in Table 1. Spiked sample solutions were freshly prepared and acidified with HCl (Fluka AG, Buchs, Switzerland) to pH of 3 to avoid decomposition prior to SPE procedure.

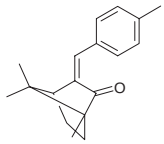
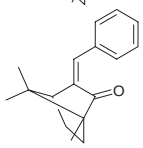
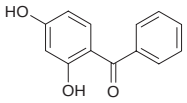
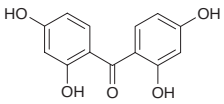
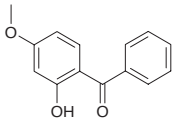
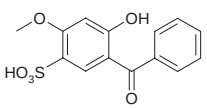
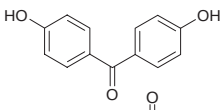
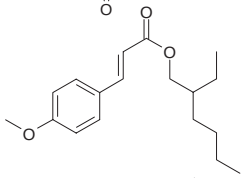
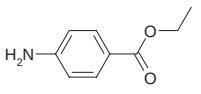
Experimental water samples were repeatedly taken during exposure experiments with frog and fish, collected in dark glass bottles, acidified with HCl to pH of 3 to avoid decomposition and stored at 4°C until immediate extraction. The animals were exposed via the aqueous route and were held in artificial tab water spiked with the test substances (subsequently referred to as experimental water, for details see (Kunz et al., 2004; Kunz et al., 2006). Precautions were taken to prevent contamination by personnel, equipment and glassware.

Separation and determination of 4-MBC and 3-BC by means of SPE and GC/MS

Solid phase extraction (SPE). One goal of this study was to develop a method to separate and concentrate residues of the UV filters 4-MBC and 3-BC, respectively, from experimental waters by solid phase extraction (SPE). Therefore commercially available SPE cartridges were tested, in order to find a cartridge which gives good separation and recoveries for 4-MBC and

Table 1

Physiochemical properties of the analysed UV filters

Compound	Chemical structure	Molecular Weight (g/kg)	Melting Point	λ_{max} (nm)	CAS
4-Methylbenzylidene camphor		254.37	66-68°C	225, 300	36861-47-9
3-Benzylidene camphor		240.34	77-81°C	223, 292	15087-24-8
Benzophenone-1		214.22	139-140°C	215, 290	131-56-6
Benzophenone-2		246.22	195°C	195, 285	131-55-5
Benzophenone-3		228.25	65°C	220, 287	131-57-7
Benzophenone-4		308.31	171°C	220, 287	4065-45-6
4,4'-Dihydroxybenzo-phenone		214.22	211-212°C	195, 297	611-99-4
Octyl-methoxycinnamate		290.40	liquid	225, 300	5466-77-3
Ethyl-4-aminobenzoate		165.19	89-92°C	221, 287	94-09-7

3-BC. Different volumes of experimental water samples and different elution solvent were tested in order to develop an optimized SPE procedure.

Gas chromatography-mass spectrometry (GC/MS). In order to determine the UV filters 4-MBC and 3-BC, the GC/MS method used by Poiger et al. (2004) was established in our laboratory. This was performed on a Saturn WS System (Varian Star 3400 CX GC, Saturn 3 MS).

Extension of the SPE method for 9 UV filters and development of a HPLC method

SPE optimisation for the extraction of 9 UV filters. In order to extract and concentrate residues of 9 UV filters from experimental waters, modifications of the SPE procedure were necessary, as the previously developed method for the extraction of 4-MBC and 3-BC failed to extract BP-4, 4-DHB, BP-2 and displayed only poor recoveries for BP-1 (Tab. 2). Hence in order to extract 9 chemically distinct compounds the SPE method was further improved. Twelve commercially available SPE cartridges were tested to find the appropriate cartridges with the best recoveries for every compound analysed.

HPLC method development for the separation of 9 UV filters. GC/MS turned out to be an adequate method for the determination of 4-MBC and 3-BC. When 7 additional UV filters were tested with GC/MS, very poor sensitivity was achieved for some of the benzophenones (see results and discussion). Therefore a HPLC method was developed for the simultaneous determination of 9 UV filters, by choosing appropriate HPLC columns, wavelengths and gradient elution. The HPLC 1100 system (Agilent Technologies, Basel, Switzerland) used in this study comprised a solvent delivery pump, an autosampler, a photodiode array detector (DAD) and chromatography software (Agilent ChemStation) for LC and LC/MS.

Calculations for Linearity, LOD and LOQ. For the GC/MS and HPLC methods regression analysis were used for linearity calculations for all 9 UV filters in the investigated concentration ranges-. The LOD for the developed GC/MS and HPLC methods was defined as the UV filter concentration that gives a signal equal to $3y_b$, where y_b is the signal of the baseline noise. Similarly, the LOQ was defined as $10y_b$.

Table 2 Recoveries with different SPE cartridges and eluents.

	Recovery (%) with different eluents								
SPE	**BP-4**	**4-DHB**	**BP-2**	**Et-PABA**	**BP-1**	**BP-3**	**3-BC**	**4-MBC**	**OMC**
	Eluent: Dichloromethane								
I 101	18.9 ± 0.1	92.8 ± 0.2	87.6 ± 0.5	47.7 ± 0.1	82.0 ± 0.3	81.6 ± 0.4	74.5 ± 0.3	71.2 ± 0.3	55.9 ± 1.4
SX US				62.3 ± 0.1	***101.9 ± 0.1***	***94.6 ± 0.1***	87.7 ± 0.1	82.9 ± 0.1	55.5 ± 0.1
SX P			3.7 ± 0.1	50.2 ± 0.1	100.5 ± 0.2	92.7 ± 0.1	78.3 ± 0.4	83.3 ± 0.4	70.8 ± 0.5
SX CM						83.0 ± 1.1	81.9 ± 1.2	***86.4 ± 0.9***	***86.5 ± 1.3***
SP C18		***105.6 ± 0.5***		***67.9 ± 0.3***	.7 ± 0.1	34.9 ± 0.1	***97.4 ± 0.4***	***92.0 ± 0.3***	69.5 ± 0.3
CE		5.4 ± 0.1		56.9 ± 0.5	59.4 ± 0.5	85.0 ± 0.8	81.9 ± 0.8	78.2 ± 0.8	62.7 ± 0.6
I C8E				56.8 ± 2.0	59.6 ± 2.2	53.8 ± 1.9	25.0 ± 0.9	38.4 ± 1.4	47.0 ± 2.9
I C18				56.6 ± 0.4	22.4 ± 0.1	65.2 ± 0.4	74.8 ± 0.5	70.7 ± 0.6	56.1 ± 2.1
S DPA				63.0 ± 0.9		91.2 ± 1.2	83.8 ± 1.2	82.8 ± 1.2	69.7 ± 1.0
CE H				62.7 ± 2.1	58.0 ± 1.9	86.4 ± 2.7	82.5 ± 2.6	78.5 ± 2.5	62.3 ± 2.1
S Env C						84.3 ± 3.6	79.3 ± 3.4	80.7 ± 3.5	67.8 ± 3.0
I C18E					30.5 ± 0.1	88.4 ± 0.1	***89.2 ± 0.1***	***87.1 ± 0.1***	83.8 ± 0.1
I PAH				24.8 ± 0.1	6.0 ± 0.1	15.5 ± 0.1	8.0 ± 0.1	8.6 ± 0.1	4.9 ± 0.3
	Eluent: Methanol								
I 101	74.2 ± 0.3	85.7 ± 0.5	78.1 ± 0.4	46.0 ± 0.2	84.8 ± 0.4	76.3 ± 0.2	34.3 ± 0.2	46.8 ± 0.1	24.7 ± 0.1
I C18	74.2 ± 2.0	76.3 ± 1.5	74.7 ± 1.5	27.4 ± 0.5	75.0 ± 1.6	41.0 ± 0.8	18.7 ± 0.4	24.8 ± 0.5	19.4 ± 0.4
I E+	29.7 ± 1.3	92.7 ± 1.6	88.2 ± 1.8		86.4 ± 1.7	23.4 ± 0.6	43.3 ± 0.8	35.7 ± 0.7	3.5 ± 0.1
S NH2		8.4 ± 0.1			1.1 ± 0.1		2.5 ± 0.1	6.0 ± 0.1	11.1 ± 0.1
CE		96.4 ± 0.5	90.7 ± 0.5	39.9 ± 0.2	93.2 ± 0.5	57.7 ± 0.4	29.9 ± 0.2	26.0 ± 0.1	18.2 ± 0.1
SP C18	***104.3 ± 0.1***	***106.9 ± 0.4***	***102.4 ± 0.4***	.9 ± 0.2	**104.2 ± 0.4**	64.2 ± 0.1	47.0 ± 0.2	36.2 ± 0.1	24.1 ± 0.1
	←polar ---------- non-polar →								

Tested SPE cartridges with corresponding mean recoveries and standard deviations for 9 UV filters, based on spiked water samples with compound concentrations of 10 µg/L. The elution solvents used were either dichloromethane ($MeCl_2$) or methanol (MeOH). Cartridges used: Isolute SPE 101 (I 101), Strata-X 33u Universal Sorbent (SX US), Strata-X-C Polymeric Sorbent (SX-C P), Strata-X-C 33um Cation Mixed-Mode Polymer (SX-CM), Sep-Pak Vac 3cc C18 (SP C18), Chromabond Easy (CE), Isolute C8/ENV$^+$ (I C8E), Isolute C18(EC) (I C18), Supelco Discovery DPA-6S (S DPA), Chromabond Easy HRP (CE H), Supelco Supeclean ENVI-Carb (S ENV), Isolute C18/ENV$^+$ (I C18E), Isolute ENV$^+$ (I E+), Isolute PAH (I PAH), Supelco Discovery DSC-NH_2 (S NH2). In bold are the cartridges used for optimised SPE procedures and subsequent recoveries.

Results

The aim of this study was to develop one analytical method that allows the determination of 9 commonly used UV filters with large differences in polarity and water solubility in experimental waters originating from exposure experiments with aquatic organisms. Therefore we first modified an existing method (Poiger et al., 2004; Balmer et al., 2005) based on a solid phase extraction procedure coupled with GC/MS in order to analyse 4-MBC and 3-BC in water samples from exposure experiments. Second we extended the SPE method to additional 7 commonly used UV filters and developed a HPLC method for 9 UV filters.

Separation and determination of 4-MBC and 3-BC by means of SPE and GC/MS

Solid phase extraction (SPE). For the extraction of 4-MBC and 3-BC from experimental water we used commercially available SPE cartridges instead of glass columns containing polystyrene adsorbent. We evaluated three different SPE cartridges for this purpose, Isolute C8/ENV, C18/ENV and C18/PAH. In Table 2 the achieved recoveries are listed. For further analysis the Isolute C18/ENV$^+$ cartridges were chosen, because of good recoveries from spiked water samples between 87% and 89% (Tab. 2) and their broad use and applicability.

The extraction of 4-MBC and 3-BC, respectively, from experimental waters was performed as follows: Isolute C18/ENV$^+$ cartridges (Separtis GmbH, Grellingen, Switzerland). C18/ENV$^+$ cartridges were conditioned with 2 x 3 mL dichloromethane, followed by the same amount of methanol and H_2O bi-distilled prior to the extraction. Then the experimental water samples (usually 250 mL) were filtered under vacuum through the cartridges at a flow rate of 1-2 mL/min. The cartridges were then air-dried for 30 min and eluted into test tubes with 3 x 3 mL dichloromethane. The test tubes were transferred to a Syncore Reactor (Büchi, Flawil, Switzerland) and dichloromethane was reduced to dryness under vacuum (700 mbar, 50 °C). The glass walls were then rinsed with 1 mL of dichloromethane in order to increase the recovery and then dichloromethane was allowed to evaporate to dryness under the same conditions. The dry eluent was resuspended in 100 µL of 2-hexanol and was then transferred into a GC vial (brown glass) and analysed with GC/MS by on column injection.

With this SPE method we achieved a simplification of the extraction procedure and a better signal/noise ratio (data not shown). The cleanup step used by Poiger et al. (2004) after SPE was abandoned, once we had demonstrated that it did not improve recoveries or determination by GC/MS. For a internal standard we first used benzyl cinnamate, which gave good results when working with spiked water samples, but was no longer detectable by GC/MS when added to

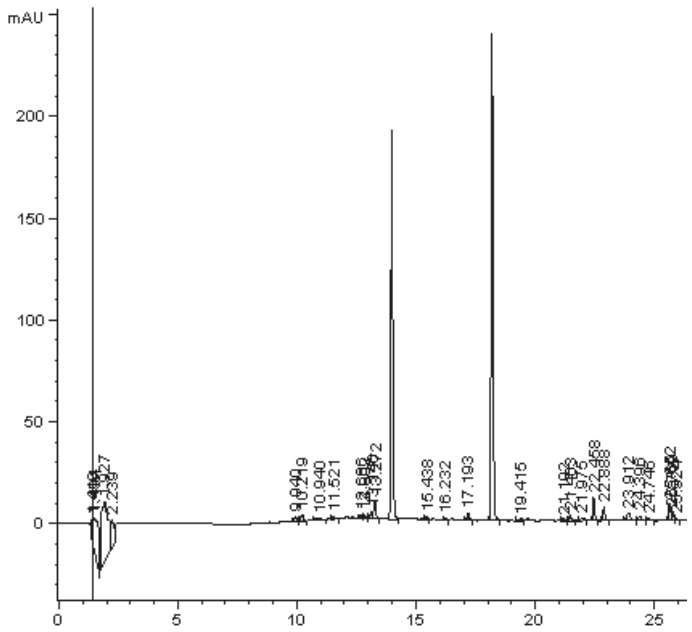


Figure. 1 Typical GC/MS-SIM chromatogram of 4-methylbenzylidene camphor (4-MBC) and 3-benzylidene camphor (3-BC) extracted from experimental water samples of exposure experiments with fish. Nominal concentrations for both compounds were 500 µg/L. Water samples were taken directly after water renewal (0 h) and before the next renewal after 24 hours of exposure (24 h). Actual concentrations were determined by means of an external standard with the same concentration as the experimental water sample (500 µg/L).

our experimental water samples. Instead, we used the other analyte as external standard (e.g. for the analysis of 4-MBC, 3-BC served as external standard). Thereby the amount of UV filter was determined from peak area relative to the internal standard (Fig. 1).

Gas chromatography-mass spectrometry (GC/MS). The analysis of 4-MBC and 3-BC was performed on a Saturn WS System (Varian Star 3400 CX GC, Saturn 3 MS) by on column injection (SPI). The GC was fitted with a SE 54 capillary column (J&W Scientific, MSP Friedli + Co., Koeniz, Switzerland), 30 m x 0.32 mm x 0,25 µm. Analyses were performed in the selected ion monitoring (SIM) mode using electron ionisation (EI). The following ions were selected from the spectrum of each compound in order to quantify the response under SIM mode: *m/z* 239, 240 and 241 for 3-BC, *m/z* 239, 253, 254 and 255 for (Z-)-4-MBC, *m/z* 239, 253, 254 and 255 for (E-)-4-MBC. The temperature program run was: 85°C, held for 2 min, ramped at 20 °C/min to 180 °C for 4.75 min and then ramped at 5 °C/min to 280 °C for 20 min. The injector temperature program run was: 60 °C, held for 0.1 min, ramped at 180 °C/min to 280 °C, held for 20 min and 2.0 µL injections were made. Helium (Carbagas, Bern, Switzerland, purity grade > 99.99%) was used as carrier gas with an inlet pressure of 19 psi (1.34 bar).

SPE optimisation for the extraction of 9 UV filters. The cartride (C18/ENV$^+$) used to extract 4-MBC and 3-BC cartridge failed to extract BP-4, 4-DHB, BP-2 and displayed only poor recoveries for BP-1 (Tab. 2). Thus we tested twelve commercially available SPE cartridges in order to find the appropriate cartridges with the best recoveries for every compound analysed (Tab. 2). The Sep Pak C18 cartridge (Waters AG, Rupperswil, Switzerland) was able to extract 7 out of 9 compounds, with recoveries between 102 and 107% for BP-4, 4-DHB, BP-2 and BP-1 when eluted with methanol, and between 92 and 105% for 4-DHB, 3-BC and 4-MBC when eluted with dichloromethane. For Et-PABA, the recovery was only 68% with this solvent and could not be improved by applying other cartridges. Because of the insufficient recoveries with Sep Pak C18 cartridges, UV filters BP-3 and OMC were extracted with either Strata-X Universal Sorbent cartridges or Strata X Cation Mixed Mode cartridge (both Brechbuehler AG, Schlieren, Switzerland), respectively, and dichloromethane as elution solvent resulting in recoveries of 87 and 95% (Tab. 2). The dry eluent was resuspended in ethanol prior to HPLC analysis instead of 2-hexanol, leading to improved peak quality.

Upgrading of the GC/MS method for 9 UV filters. We expanded the GC/MS method in order to determine additional 6 UV filters (BP-1, BP-2, BP-3, BP-4, DHB, Et-PABA and OMC). This led to difficulties for BP-1, which gave very broad peaks and for BP-2 and DHB, which were not detectable in GC/MS and led to artefacts (data not shown). Modifications of temperature regime, injection temperatures and solvents did not improvement the detection. Thus experiments were conducted to analyse the compounds by HPLC instead of applying GC/MS.

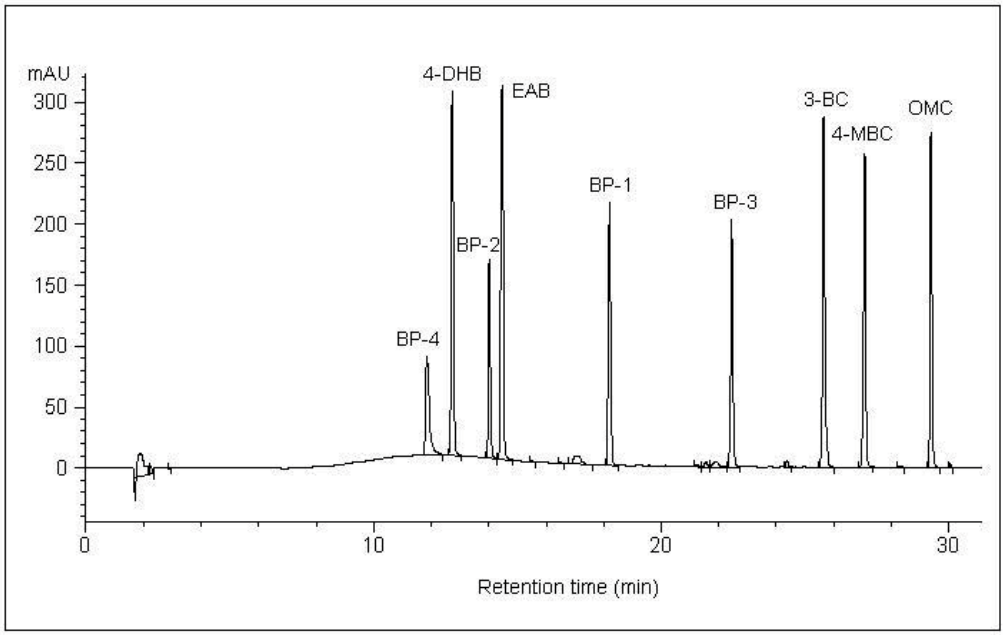


Figure 2 Typical chromatogram of the 9 UV filters determined by HPLC-DAD (290 nm), showing peaks of the compounds solved in ethanol (25 mg/L). This concentration is based on a concentration factor of 2500 when concentrating 250 mL of a spiked water sample (10 µg/L) by SPE to 100 µL of eluent.

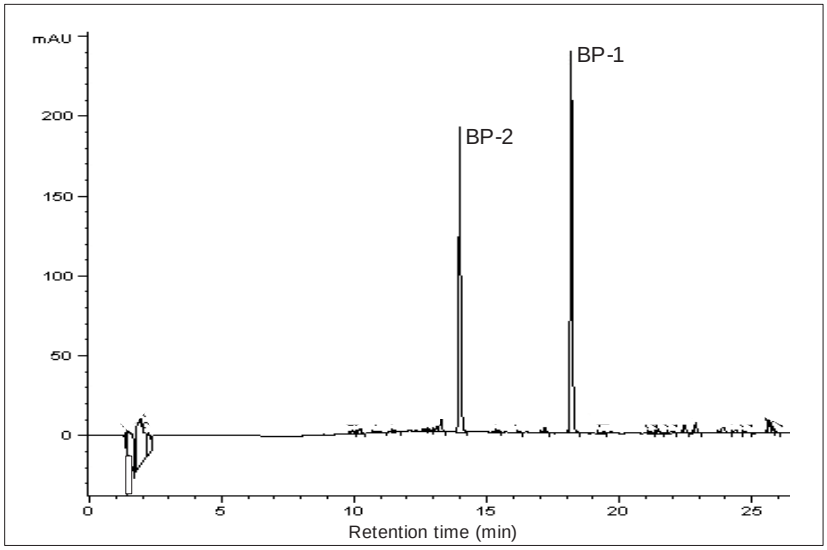


Figure 3 Typical HPLC-DAD (290 nm) chromatogram of determination of benzophenone-2 (BP-2) extracted from experimental water samples of exposure experiments with fish. Nominal concentrations of benzophenone-1 (BP-1) and benzophenone-2 (BP-2) were 10 µg/L. Water samples were taken directly after water renewal (0 h) and before the next renewal after 24 hours of exposure (24 h). Actual concentrations of BP-2 were determined by means of BP-1 as internal standard.

HPLC method development and optimisation for 9 UV filters. For the HPLC method development, data collection was first performed for all 9 compounds across the 190-400 wavelength range. The diode array detector was then set to 290 nm, which is a compromise giving good UV responses for all 9 analytes (Fig. 2). Bi-distilled water was processed

as procedural blank and spiked with UV filters at concentrations of 10 and 100 µg/L for the determination of recoveries. As internal standards we used UV filters with structural or chemical similarities to the UV filter analysed (Tabs. 1 and 2). The amount of UV filter was determined from peak area ratios relative to the internal standard (Fig. 3).

Linearity, LOD and LOQ for GC/MS and HPLC. Linearities for all 9 compounds in the investigated concentration ranges are presented in Table 3. For the developed GC/MS method the LOD and LOQ for 4-MBC and 3-BC were in the range 50–62.5 µg/L and 167.5–212.5 µg/L, respectively. When including SPE in these calculations with a concentration factor of 2'500, LOQ of 67-85 ng/L were achieved for 4-MBC and 3-BC, respectively. For the HPLC method for 9 UV filters the LOD and LOQ values ranged between 87.5-137.5 µg/L and 287.5–462.5 µg/L, respectively, for all compounds except BP-4, which had higher LOD and LOQ. Again by including SPE with a concentration factor of 2'500 in these calculations, LOQ of 115-185 ng/L were achieved for all compounds except BP-4 (Tab. 3).

Table 3 Analytical parameters of calibration curves, LOD and LOQ.

UV filter	RT (Min) standard	CR (µg/L)	Regression Equation[b] Intercept[c]	Slope[d]	r^2 (*n*)[e]	LOD (µg/L)	LOQ (µg/L)	SPE LOQ (ng/L)	Internal
GC/MS									
3-BC	10.700	0.564-56.4	-34.81 ± 22.18[a]	12.21 ± 0.64[a]	0.9918(5)	62.5	212.5	85	4-MBC
4-MBC	11.783	0.516-51.6	-30.38 ± 18.76[a]	15.27 ± 0.59[a]	0.9909(5)	50.0	167.5	67	3-BC
HPLC									
4-MBC	26.932	0.05-100	57.42 ± 35.30	142.70 ± 0.93	0.9998(7)	100.0	325.0	130	3-BC
3-BC	25.522	0.10-100	102.40 ± 54.27	153.2 ± 1.32	0.9997(6)	100.0	312.5	125	4-MBC
BP-1	18.130	0.05-100	26.74 ± 16.63	107.8 ± 0.44	0.9999(7)	112.5	362.5	145	BP-2
BP-2	13.953	0.10-100	18.11 ± 10.20	83.34 ± 0.27	0.9999(7)	137.5	462.5	185	BP-1
BP-3	22.383	0.05-100	29.01 ± 15.69	113.40 ± 0.41	0.9999(7)	125.0	387.5	155	BP-1
BP-4	11.901	0.05-100	8.21 ± 6.21	75.19 ± 0.15	1.0000(6)	525.0	1'750.0	700	BP-1
4-DHB	12.663	0.05-100	66.79 ± 39.91	157.00 ± 1.05	0.9998(7)	87.5	287.5	115	3-BC
OMC	29.963	0.05-100	80.58 ± 46.75	153.00 ± 1.23	0.9997(7)	100.0	312.5	125	4-MBC
EAB	14.414	0.05-100	87.85 ± 55.06	173.10 ± 1.45	0.9997(7)	100.0	300.0	120	4-DHB

Retention times (RT), concentration range (CR), linearity, limits of detection (LOD) and limits of quantitation (LOQ) of the GC/MS and HPLC method. SPE LOQ Limits of quantitation including the extraction and concentration by SPE (SPE LOQ) with a concentration factor of 2'500 when concentrating 250 mL of a spiked water sample (sample concentration: 100 µg/L) by SPE to 100 µL of eluent.

[a] value = calculated value x 10^{-5}

[b] Linear unweighted regression analysis, with a regression equation $y = a + bx$, where x is concentration in µg/L.

[c] (a ± S.D.), where a is the intercept and S.D. is the standard deviation.

[d] (b ± S.D.), where a is the slope and S.D. is the standard deviation.

[e] r^2 is the correlation coefficient and n is the number of points in each calibration curve; each point is the mean of 4 experimental measurements.

Table 4. Example of Nominal and measured water concentrations of analyzed UV filters by HPLC and GC/MS (4MBC and 3BC) during a 14-day experiment with juvenile fathead minnows, exposed via the aqueous route toward 9 different UV filters (from Kunz et al. 2006).

	Exposure concentrations				
	Nominal	Measured			
	(µg/L)	0 h (µg/L)	24 h (µg/L)	Median (µg/L)*	After 24 h (%)**
3BC	10	9.5 ± 0.3	8.0 ± 1.2	9	**84%**
	500	516.7 ± 36.7	352.5 ± 130.8	435	**68%**
	1'000	1'070.0 ± 28.3	835.0 ± 49.5	953	**78%**
BP1	10	9.8 ± 0.1	8.1 ± 0.2	9	**82%**
	1'000	1'032.4 ± 54.8	930.0 ± 14.1	981	**90%**
	5'000	5'191.7 ± 58.7	4'647.1 ± 221.4	4'919	**90%**
BP2	10	10.7 ± 0.6	9.9 ± 0.9	10	**93%**
	1'000	1'102.5 ± 9.8	1'031.7 ± 17.0	1'067	**94%**
	10'000	9'747.3 ± 557.9	7'818.5 ± 104.5	8'782	**80%**
4MBC	10	9.6 ± 1.8	7.4 ± 0.4	9	**77%**
	500	492.4 ± 102.7	337.5 ± 17.7	415	**69%**
	1'000	826.1 ± 189.4	680.0 ± 198.0	753	**82%**
OMC	10	8.8 ± 0.1	6.5 ± 0.7	8	**74%**
	1'000	1'012.5 ± 165.6	765.0 ± 63.6	889	**76%**
	5'000	5'450.0 ± 282.8	4'600.0 ± 141.4	5'025	**84%**
BP3	10	13.5 ± 1.1	9.7 ± 0.2	12	**71%**
	1'000	879.5 ± 115.0	652.2 ± 65.4	766	**74%**
	5'000	4'175.0 ± 247.5	3'625.0 ± 106.1	3'900	**87%**
BP4	10	11.5 ± 0.9	10.8 ± 0.8	11	**94%**
	1'000	1'068.2 ± 64.4	1'027.7 ± 99.2	1'048	**96%**
	5'000	5'158.9 ± 425.2	4'634.3 ± 26.4	4'897	**90%**
4DHB	10	11.8 ± 1.0	9.0 ± 0.1	10	**76%**
	1'000	901.9 ± 48.4	899.0 ± 42.9	900	**100%**
	5'000	5'388.5 ± 2.7	4'633.0 ± 232.4	5'011	**86%**

*: Median of actual concentrations at 0 h and 24h
**: Percentage of actual concentration at 24 h relative to 0h

Analysis of experimental waters

The methods described were applied for the GC/MS or HPLC determination, respectively, of actual concentrations of 2 UV filters in experimental waters with frogs (for details see (Kunz et al., 2004)) and of 9 UV filters in experimental waters with fish. The results are shown in Table 4 and details are given in Kunz et al. (2006)(Fig. 3).

Discussion

The aim of this study was to develop one analytical method that allows the determination of 9 commonly used UV filters with large differences in polarity and water solubility in experimental waters originating from exposure experiments with aquatic organisms. Two methods for analysis of UV filters in water were developed. In order to analyse 4-MBC and 3-BC in water samples from exposure experiments we modified an existig method (Poiger et al., 2004; Balmer et al., 2005) based on a solid phase extraction procedure coupled with GC/MS. The extension of this method to additional 7 commonly used UV filters led to difficulties, and therefore, resulted in the development of a SPE and HPLC-DAD method for 9 UV filters.

Modification and optimisation of SPE and GC/MS method for 4-MBC and 3-BC. Because of slight differences in the requirements the existing method (Poiger et al., 2001; Poiger et al., 2004) was simplified as follows. Extraction of 4-MBC and 3-BC from experimental water was performed with commercially available SPE cartridges instead of glass columns containing polystyrene adsorbent. For this purpose we tested three different SPE cartridges (Isolute C8/ENV, C18/ENV and C18/PAH) and finally the Isolute C18/ENV$^+$ cartridges was chosen for extraction of 4-MBC and 3-BC, because of its good recoveries and its broad use and applicability. This led to a simplification of the extraction procedure.

We abandoned the cleanup of the extracts on silica after SPE (Poiger et al., 2001; Poiger et al., 2004), once we had demonstrated that the cleanup, besides removing some other compounds and leading to artefacts in the cleaned extract, was not advantageous for the GC/MS determination of 4-MBC and 3-BC in experimental water, although it might be still of use in natural waters. Furthermore the internal standard compound was changed. While benzyl cinnamate as internal standard gave good results in our hands when working with spiked water samples, it was no longer detectable by GC/MS when added to our experimental water samples. Instead, we used the other analyte as internal standard (e.g. for the analysis of 4-MBC, 3-BC served as internal standard, and vice versa). These modifications led to a more practicable and faster SPE procedure and adaptations of the GC/MS measurement regime.

Difficulties with the upgrading of the GC/MS method for 9 UV filters. The expansion of the GC/MS method in order to determine additional 6 UV filters (BP-1, BP-2, BP-3, BP-4, DHB, Et-PABA and OMC) led to difficulties. BP-1 gave very broad peaks, BP-2 and DHB were not detectable and led to artefacts. Modifications of temperature regime, injection temperatures and solvents did not lead to improvements in detection. Possibly, high boiling points (DHB, 213-215°C), reduced volatility and enhanced polarity made them difficult to determine by GC/MS. Thus experiments were conducted to analyse the compounds by HPLC instead of applying

GC/MS. As a consequence of the difficulties in upgrading the SPE and GC/MS method for 4-MBC and 3-BC, we developed a novel SPE and HPLC method in order to determine effective concentrations of 9 different UV filters in experimental exposure waters.

SPE optimisation for the extraction of 9 UV filters. The previously used C18/ENV$^+$ cartridge, which gave good recoveries for 4-MBC and 3-BC failed to extract BP-4, 4-DHB, BP-2 and displayed only poor recoveries for BP-1. Thus adaptation of the SPE method had to be undertaken to extract 9 chemically distinct compounds. From twelve commercially available SPE cartridges tested three were chosen for further SPE of UV filters. The Sep Pak C18 cartridge (Waters AG, Rupperswil, Switzerland) was used for the extraction of BP-4, 4-DHB, BP-2 and BP-1 with methanol as eluted and for 4-DHB, 3-BC and 4-MBC with dichloromethane as eluent. For Et-PABA, the recovery was only 68% with this solvent and could not be improved by applying other cartridges. Because of the insufficient recoveries with Sep Pak C18 cartridges, UV filters BP-3 and OMC were extracted with either Strata-X Universal Sorbent cartridges or Strata X Cation Mixed Mode cartridge (both Brechbuehler AG, Schlieren, Switzerland), respectively and dichloromethane as elution solvent. The dry eluent was resuspended in ethanol prior to HPLC analysis instead of 2-hexanol, leading to improved peak quality.

HPLC method development and optimisation for 9 UV filters. In order to measure the nine UV filters by HPLC, the diode array detector was set to 290 nm, which is a compromise giving good UV responses for all 9 analytes (Fig. 2). For analysis of experimental water samples a guard cartridge was not needed, but it might be necessary when analysing natural waters.

As internal standards we used UV filters with structural or chemical similarities to the UV filter analysed (Tabs. 1 and 2). For example, when determining residues of 4-MBC in experimental waters, 3-BC was used as internal standard. The amount of UV filter was determined from peak area ratios relative to the internal standard (Fig. 3). This faster and more feasible procedure comprises of a SPE method using three different cartridges (Sep-Pak Vac C18, Strata-X Universal Sorbent and Strata-X-C Cation Mixed-Mode Polymer), two elution-solvents (methanol and dichloromethane) and a HPLC-DAD method for simultaneous analysis of these 9 compounds.

Linearity, LOD and LOQ for GC/MS and HPLC. Regression analysis revealed linearity for all 9 compounds in the investigated concentration ranges (Tab. 3). For the developed GC/MS method the LOD and LOQ for 4-MBC and 3-BC were in the low µg/L range. When including SPE in these calculations with a concentration factor of 2'500, LOQ in the low ng/L range were achieved for 4-MBC and 3-BC, respectively

For the newly developed HPLC method for 9 UV filters the LOD and LOQ, were a little higher than for GC/MS, and were in the low to medium µg/L range for all compounds except BP-4,

which had higher LOD and LOQ. Again by including SPE with a concentration factor of 2'500 in these calculations, LOQ of 115-185 ng/L were achieved for all compounds except BP-4 (Tab. 3). Our method may be used for water samples with lower concentrations by increasing volumes for SPE to 500 or 1000 mL and reducing the final eluent volume to 50 µL, thereby concentration factors of 5'000 to 10'000 may be achieved.

Application

In this study, an analytical method for the detection of the UV filters 4-MBC and 3-BC based on SPE and GC/MS was modified and simplified. In addition, a SPE-HPLC method for the quantitative determination of 9 commonly used UV filters in experimental waters was developed. Accordingly, these 9 chemically different compounds were extracted and concentrated from spiked water samples by the use of 3 different cartridges and two different sovents, giving recoveries higher than 87 % for all 8 UV filters. The resulting extracts were either analysed by GC/MS (4-MBC and 3-BC) or HPLC (all 9 compounds). Although HPLC-DAD does not achieve the sensitivity of GC/MS or HPLC/MS, it offers a fast and reliable analysis of experimental water samples in aquatic toxicological studies.

We applied the methods described for the determination of actual concentrations of 2 UV filters in experimental waters with frogs (for details see Kunz et al. (2004)) and of 9 UV filters in experimental waters with fish (Kunz et al., 2006) (Tab. 4 and Fig. 3) and achieved good results. However, it may well be used as a versatile tool to determine UV filter residues in natural waters. Especially when larger samples volumes are used for the SPE procedure, the detection limit will be reduced to the low ng/L range. This is particularly needed for monitoring purposes, as UV filters are of growing concern regarding their possible hormonal activity and adverse effects on the aquatic environment.

Acknowledgements

This work was supported by the Swiss National Science Foundation (NFP 50, contract 4050-66554 to K. Fent). We thank D. de Bryn and Th. Purtschert for valuable contributions to the project; we also thank A. Buettler and B. Gygax, for technical support and valuable advice (all at the University of Applied Sciences Northwestern Switzerland, Department of Chemistry and Bioanalytics) and F. Juettner, University of Zurich, for support.

References

Abdel-Nabi, I. M., Kadry, A. M., Davis, R. A., Abdel-Rhaman, M. S., 1992. Development and validation of a high-performance liquid chromatography method for the determination of benzophenone-3 in rats. Journal of Applied Toxicology 12(4), 255-259.

Balmer, M., Buser, H. R., Müller, M. D., Poiger, T., 2004. Occurence of the organic UV filter compounds BP-3, 4-MBC, EHMC and OC in wastewater, surface waters, and in fish from Swiss lakes. Wädenswil, FAW**:** 50.

Balmer, M., Buser, H. R., Müller, M. D., Poiger, T., 2005. Occurrence of some organic UV filters in wastewater, in surface waters, and in fish from Swiss lakes. Environ. Sci. Technol. 39, 953-962.

Buser, H. R., Balmer, M. E., Schmid, P., Kohler, M., 2006. Occurence of UV filters 4-methylbenzylidene camphor and octocrylene in fish from various swiss rivers with inputs from wastewater treatment plants. Environ. Sci. Technol. 40, 1427-1431.

Chisvert, A., Pascual-Marti, M. C., Salvador, A., 2001. Determination of the UV-filters worldwide authorised in sunscreens by high-performance liquid chromatography. Use of cyclodextrins as mobile phase modifier. Journal of Chromatography A 921, 207-215.

Chisvert, A., Salvador, A., 2002. Determination of water-soluble UV-filters in sunscreen sprays by liquid chromatography. Journal of Chromatography A 977, 277-280.

DiNunzio, J., Gadde, R. R., 1990. Determination of sunscreen compounds in topical sunscreen products. Journal of Chromatography 519, 117-124.

Felix, T., Hall, B. J., Brodbelt, J. S., 1998. Determination of benzophenone-3 and metabolites in water and human urine by solid-phase microextraction and quadropole Ion trap GC-MS. Analytica Chimica Acta 371, 195-203.

Gagliardi, L., Cavazzutti, G., Montarella, L., Tonelli, D., 1989. Determination of sun-screen agents in cosmetic products by reversed-phase high-performance liquid chromatography. Journal of Chromatography 464, 428-433.

Giokas, D. L., Sakkas, V. A., Albanis, T. A., 2004. Determination of residues of UV filters in natural waters by solid-phase extraction coupled to liquid chromatography-photodiode array detection and gas chromatography-mass spectrometry. Journal of Chromatography A 1026, 289-293.

Hauri, U., Lütolf, B., Hohl, C., 2003. Determination of organic sunscreen filters in cosmetics with HPLC/DAD. Mitteilungen Lebensmitteluntersuchung Hygiene 94, 80-92.

Holbech, H., Norum, U., Korsgaard, B., Bjerregaard, P. (2002). The chemical UV-filter 3-benzylidene camphor causes an estrogenic effect in an *in vivo* fish assay. SETAC Europe 12th Annual Meeting, Wien.

Inui, M., Adachi, T., Takenaka, S., Inui, H., Nakazawa, M., Ueda, M., Watanabe, H., Mori, C., Iguchi, T., Miyatake, K., 2003. Effect of UV screens and preservatives on vitellogenin and choriogenin production in male medaka (*Oryzias latipes*). Toxicology 194, 43-50.

Kunz, P. Y., Galicia, H. F., Fent, K., 2004. Assessment of hormonal activity of UV filters in tadpoles of frog *Xenopus laevis* at environmental concentrations. Marine Environmental Research 58, 431-435.

Kunz, P. Y., Galicia, H. F., Fent, K., 2006. Comparison of *in vitro* and *in vivo* estrogenic activity of UV filters in fish. Toxicol. Sci. 90, 349-361.

Loraine, G. A., Pettigrove, M. E., 2006. Seasonal variations in concentrations of pharmaceuticals and personal care products in drinking water and reclaimed wastewater in southern california. Environ. Sci. Technol. 40, 687-695.

Nagtegaal, M., Ternes, T. A., Baumann, W., Nagel, R., 1997. UV-Filtersubstanzen in Wasser und Fischen. UWSF-Z Umweltchem Oekotox 9, 79-86.

Plagellat, C., Kupper, T., Furrer, R., de Alencastro, L. F., Grandjean, D., Tarradellas, J., 2006. Concentrations and specific loads of UV filters in sewage sludge originating from monitoring network in Switzerland. Chemosphere 62(6), 915-925.

Poiger, T., Buser, H. R., Balmer, M., Bergqvist, P. A., Müller, M. D., 2004. Occurence of UV filter compounds from sunscreens in surface waters: regional mass balace in two Swiss lakes. Chemosphere 55, 951-963.

Poiger, T., Buser, H. R., Müller, M. D., 2001. Verbrauch, Vorkommen in Oberflächengewässern und Verhalten in der Umwelt von Substanzen, die als UV-Filter in Sonnenschutzmitteln eingesetzt werden. Wädenswil, Eidg. Forschungsanstalt für Obst-, Wein- und Gartenbau, CH-8820 Wädenswil**:** 46.

Rastogi, S. C., Jensen, G. H., 1998. Indentification of UV filters in sunscreen products by high-performance liquid chromatography-diode-array detection. Journal of Chromatography A 828, 311-316.

Salvador, A., Chisvert, A., Jaime, A., 2005. Near-critical carbon dioxide extraction and liquid chromatography determination of UV filters in solid cosmetic samples: a green analytical procedurs. J. Sep. Sci. 17, 2319-2324.

Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B., Lichtensteiger, W., 2001. In vitro and in vivo estrogenicity of UV screens. Environmental Health Perspectives 109, 239-244.

Schlumpf, M., Schmid, P., Durrer, S., Conscience, M., Maerkel, K., Henseler, M., Gruetter, M., Herzog, I., Reolon, S., Ceccatelli, R., Faass, O., Stutz, E., Jarry, H., Wuttke, W., Lichtensteiger, W., 2004. Endocrine acitivity and developmental toxicity of cosmetic UV filters - an update. Toxicology 205, 113-122.

Soeberg, T., Ganderup, N. C., Kristensen, J. H., Bjerregaard, P., Pedersen, K. L., Bollen, P., Hansen, S. H., Halling-Soerensen, B., 2006. Distribution of the UV filter 3-benzylidene camphor in rat following topical application. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 834(1-2), 117-121.

Solomon, G. M., Weiss, P. M., 2002. Chemical contaminants in breast milk: time trends and regional variability. Environmental Health Perspectives 110(6), A339-A346.

Vanquerp, V., Rodriguez, C., Coiffard, C., Coiffard, L. J. M., De Roeck-Holtzhauer, Y., 1999. High-performance liquid chromatographic method for the comparison of the photostability of five sunscreen agents. Journal of Chromatography A 832, 273-277.

Chapter 5

Comparison of *in vitro* and *in vivo* estrogenic activity of UV filters in fish

Petra Y. Kunz, Hector F. Galicia and Karl Fent

published in *Toxicological Sciences, 2006, 90, 349-361*

Abstract

In this work, we evaluate whether *in vitro* systems are good predictors for *in vivo* estrogenic activity in fish. We focus on UV filters being largely used in sunscreens and in UV stabilization of materials. First, we determined the estrogenic activity of 23 UV filters and one UV filter metabolite employing a recombinant yeast carrying the estrogen receptor of rainbow trout (rtERα) and made comparisons with yeast carrying the human hERα for receptor specificity. Benzophenone-1 (BP1), benzophenone-2 (BP2), 4,4-dihydroxybenzophenone, 4-hydroxybenzophenone, 2,4,4-trihydroxy-benzophenone and phenylsalicylate showed full dose-response curves with maximal responses of 81-115%, whereas 3-benzylidene camphor (3BC), ocylsalicylate, benzylsalicylate, benzophenone-3 and benzophenone-4 displayed lower maximal responses of 15-74 %. Whereas the activity of 17β-estradiol was lower in the rtERα than the hERα, the activities of UV filters were similar or relatively higher in rtERα, indicating different relative binding activities of both ER. Subsequently, we analysed whether the *in vitro* estrogenicity of 8 UV filters is also displayed *in vivo* in fathead minnows by the induction potential of vitellogenin after 14 days of aqueous exposure. Of the three active compounds *in vivo*, 3BC induced vitellogenin at lower concentrations (435 µg/L) than BP1 (4919 µg/L) and BP2 (8783 µg/L). The study shows for the first time estrogenic activities of UV filters in fish both *in vitro* and *in vivo*. Thus we propose that receptor-based assays should be used for *in vitro* screening prior to *in vivo* testing, leading to environmental risk assessments based on combined, complementing and appropriate species-related assays for hormonal activity.

Introduction

Numerous studies have focused on compounds that are agonists for estrogen receptors α and β (ERα, ERβ) (Routledge and Sumpter 1997; Schultz *et al.* 2000; Sohoni and Sumpter 1998; Soto *et al.* 1991). These include a wide range of different compounds having different molecular structures and binding affinities that may induce effects in wildlife (Jobling *et al.* 1998; Vos *et al.* 2000). With regard to the considerable number of chemicals that are and will be assessed for possible estrogenic activity, *in vitro* systems play an important role for identification and first screening of estrogenic compounds that interact with the ER. Within the framework on the Endocrine Disrupters Testing and Assessment (EDTA) of the OECD, animal and non-animal tests are proposed (OECD 2002). For assessing possible ecotoxicological effects, a fish *in vivo* screening assay is planned, but no *in vitro* assays using fish-based systems (OECD 2004). Considering the vast number of compounds to be tested for aquatic systems, it is important to employ appropriate *in vitro* systems for fish (Ackermann *et al.* 2002; Le Guével and Pakdel 2001; Pakdel *et al.* 2000). In this regard, the question arises as to what extend *in vitro* systems can mimic *in vivo* activity of estrogenic compounds. *In vitro* systems are cost effective tools and allow for rapid screening of a large number of compounds, but they have limitations, which may result in unreliable predictions. Therefore, the most appropriate way to determine the endocrine-disrupting activity of chemicals seems to include both *in vitro* and *in vivo* assays, as no single assay may be best suited to determine the hormonal activity of a compound. In our present work, we address this question focusing on important chemicals in personal care products found in the aquatic environment.

Sunscreens and cosmetics including lipsticks, skin lotions, hair sprays, hair dyes, shampoos, and numerous other products contain increasing amounts of compounds protecting from ultraviolet (UV) radiation. Either organic UV filters, or inorganic micropigments (ZnO, TiO2) scattering and reflecting UV light, or combination of both, are applied. Increased sunlight protection factors are being used for preventing negative effects on the human skin, which generally requires higher percentages of UV filters in the products. Combinations of different UV filters are increasingly employed for absorbing UVA, UVB and UVC light.
Inputs of UV filters into the aquatic system occur directly via recreational activities (bathing) into surface water, and indirectly via wastewater. UV filters are photostable, many of them highly lipophilic (log Kow 3-7) and relatively stable in the aquatic environment (Balmer *et al.* 2005; Poiger *et al.* 2004), which makes these compounds critical for bioaccumulation. Residues of several UV filters have yet been detected in human milk (Hany and Nagel 1995) and fish (Balmer *et al.* 2005; Nagtegaal *et al.* 1997), in the latter between 21-3100 ng/g lipid, but also in lakes and wastewater, with maximum concentrations up to 125 ng/L (Poiger *et al.* 2004) and 19 μg/L (Balmer *et al.* 2005), respectively.

At present, the estrogenicity of UV filters in fish remains elusive and the ecotoxicological risk for aquatic life is not known. Estrogenic activity *in vitro* has been shown for some UV filters in MCF-7 cells (Schlumpf *et al.* 2001), recombinant cell lines (Mueller *et al.* 2003; Schreurs *et al.* 2002) and recombinant yeast systems carrying the human ERα (Kunz and Fent submitted; Routledge and Sumpter 1997; Schultz *et al.* 2000). Estrogenic activity has also been observed experimentally *in vivo* in rats (Durrer *et al.* 2005; Schlumpf *et al.* 2001; Seidlová-Wuttke *et al.* 2004). In fish high concentrations of 3-benzylidene camphor, 4-methyl-benzylidene camphor and octyl-methoxycinnamate (Holbech *et al.* 2002; Inui *et al.* 2003) were found to be estrogenic after short-term exposure. Contrary to these studies, no estrogenicity was observed at 10 µM octyl-methoxycinnamate, benzophenone-3, homosalate, octyl dimethyl-p-aminobenzoic acid, butyl methoxydibenzoylmethane and 1 µM 4-methyl-benzylidene camphor after short-term exposure of transgenic zebrafish (Schreurs *et al.* 2002). Therefore the estrogenic activity of UV filters at low aqueous concentrations remains unclear.

The objectives of this study were to elucidate, whether commonly used UV filters are estrogenic *in vitro* and *in vivo* in fish, to compare the *in vitro* activities in two *in vitro* systems carrying either a fish or the human ERα, and to compare the *in vitro* with the *in vivo* activity. We test the hypothesis that the estrogenic activity of chemicals is best assessed by the use of a tiered approach using a combination of *in vitro* and *in vivo* assays of the same phyla. As the rtERα has a different activity towards known estrogenic compounds than the hER (Le Guével and Pakdel 2001; Pakdel *et al.* 2000; Petit *et al.* 1995), the question arises whether fish-based *in vitro* systems should be used for assessing estrogenicity in fish. Direct comparison of fish *in vitro* and *in vivo* activity demonstrates that the estrogenic activity *in vivo* may be in part predictable from the *in vitro* activity, although *in vitro* screening tends to overestimate the number of estrogenic compounds due to lack of or low metabolism. This indicates the need for a tiered approach combining *in vitro* and *in vivo* assessments of hormonal activity of UV filters for ecological risk assessment.

Material and Methods

Chemicals

17β-Estradiol (E2) was purchased from Fluka AG (Buchs, Switzerland). UV filters (Tab. 1) were obtained as follows. Benzophenone-1 (BP1), benzophenone-2 (BP2), benzophenone-3 (BP3), benzophenone-4 (BP4), 4'-hydroxybenzophenone (4HB), 4,4'-dihydroxybenzophenone (4DHB), 2,4,4'-trihydroxybenzophenone (THB), 4-aminobenzoic acid (PABA), benzylsalicylate

(BS), phenlysalicylate (PS), octyl salicylate (OS), octocrylene (OC) and octyl dimethyl PABA (OD-PABA) were from Fluka AG; octyl-methoxycinnamate (OMC), 3-(4`-methylbenzylidene-

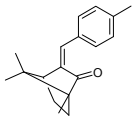
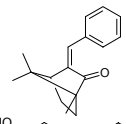
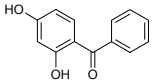
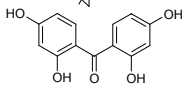
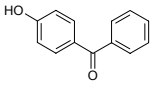
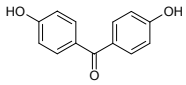
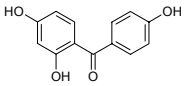
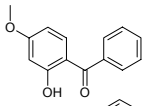
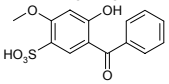
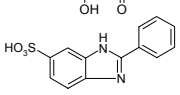
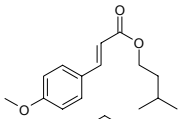
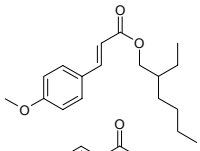
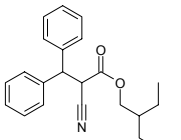
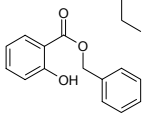
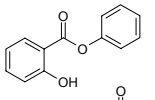
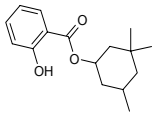
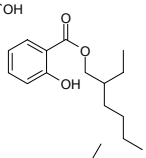
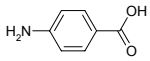
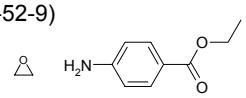
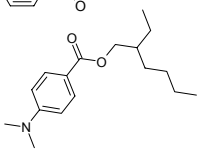
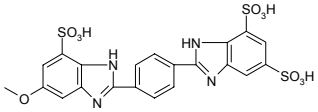
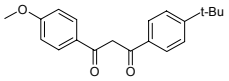
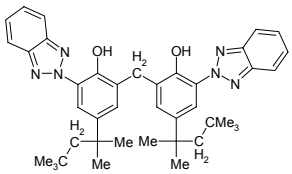
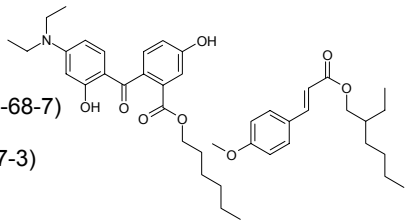
camphor) (4MBC), 3-benzylidene-camphor (3BC) and homosalate (HMS) were from Merck (Glattbrugg, Switzerland). Ethoxylated ethyl-4-aminobenzoate (PEG-25 PABA), a polymer consisting of ethyl 4-aminobenzoate and oxirane, was purchased from Induchem (Volketswil, Switzerland), and isopentyl-4-methoxycinnamate (IMC) was from Haarmann & Reimer (Holzminden, Germany). Bisimidazylate (BIM) was purchased form T.H. Geyer (Friedrichsthal, Germany). 4-tert-Butyl-4'-methoxydibenzoylmethane (BM-DBM) and 2-phenly-5-benzimidazole-sulfonic-acid (PBS) were purchased from Aldrich (Fluka AG, Buchs Switzerland). 2,2-Methylenbis-phenol (ECL) was purchased from Ciba Speciality Chemicals (Basel, Switzerland) and Uvinul A plus B (UAB), a mixture of 35% 2-(4-Diethylamino-2-hydroxybenzoyl)-benzoic acid hexylester and 65% OMC, was a gift from BASF AG (Wädenswil, Switzerland). All compounds were >99% pure. Stock solutions were made in ethanol and stored in the dark at 4°C. Analytical grade ethanol (EtOH, free of UV filters) was purchased from T.J. Baker (Stehelin AG, Basel, Switzerland). Bidestilled water was produced using a Jencons Autostill double D-ionstill destillator (Renggli AG, Rotkreuz, Switzerland).

Experiments in vitro in yeast

Recombinant yeast assay expressing the rainbow trout estrogen receptor alpha (rtERα assay). We investigated estrogenic activity of UV filters *in vitro* by applying a quantitiative β-galactosidase assay in liquid culture of recombinant yeast expressing the estrogen receptor of rainbow trout (rtERα) that was kindly provided by F. Pakdel, University of Rennes. We slightly modified the previously described assay procedure (Le Guével and Pakdel 2001; Petit *et al.* 1995). In general the assay is based on transactivation of rtERα and induction of β-galactosidase leading to a color change. The estrogen-inducible expression system used is described in detail in (Le Guével and Pakdel 2001). In brief, the yeast (*Saccharomyces cerevisiae*) genome carries a stably integrated DNA sequence of the rainbow trout estrogen receptor (rtERα). Yeast cells also contain expression plasmids carrying two estrogen responsive elements (ERE) upstream of the yeast proximal iso-1-cytochrome c gene promoter fused to the lacZ gene (encoding the enzyme β-galactosidase). Thus, the induction is strictly dependent on the presence of rtERα and estrogens (Petit *et al.* 1995). When an active ligand (i.e. 17β-estradiol or an estrogenic UV filter) binds to the receptor, β-galactosidase is synthesised and secreted into the medium, leading to a catalytic hydrolysis of o-nitrophenly-β-D-galactopyranosid (ONPG) and resulting in the development of a yellow color which was measured as absorbance at 405 nm.

Preparation of rtERα assay media and yeast growth. The assay media were prepared as previously published (Le Guével and Pakdel 2001; Petit *et al.* 1995) and described (F. Pakdel, personal communication) with the following amendments. Complete Minimal Dropout Medium (CM) was prepared by adding 2 % D-glucose instead of 1 %. In addition to the CM media we used YPD growth media (2 % peptone enzymatic digest from meat, 2 % D-glucose and 1%

Table 1. Chemical structures, molecular weight and CAS numbers of compounds analysed.

Compound MW, (CAS)	Chemical structure	Compound MW, (CAS)	Chemical structure
4MBC 254.37 (36861-47-9)		3BC 240.34 (15087-24-8)	
BP1 214.22 (131-56-6)		BP2 246.22 (131-55-5)	
4HB 198.22 (1137-42-4)		4DHB 214.22 (611-99-4)	
THB 230.22 (1470-79-7)		BP3 228.25 (131-57-7)	
BP4 308.31 (4065-45-6)		PBS 274.30 (27503-81-7)	
IMC 248.32 (71671-10-2)		OMC 290.40 (5466-77-3)	
OC 361.48 (6197-30-4)		BS 228.25 (118-58-1)	
PS 214.22 (118-55-8)		HMS 262.35 (118-56-9)	
OS 250.33 (118-60-5)		PABA 137.10 (150-13-0)	
PEG25-PABA (113010-52-9) 44.05 (75-21-8) 165.2 (94-09-7)		OD-PABA 277.41 (21245-02-3)	
BIM 275.40 (180898-37-7)		BM-DBM 310.38 (70356-09-1)	
ECL 658.87 (103597-45-1)		UAB 397.52 (302776-68-7) 290.40 (5466-77-3)	

Abbrevations: MW, molecular weight; CAS, Chemical Abstracts Service; 4MBC, 3-(4`-methylbenzylidene-camphor); 3BC, 3-benzylidene-camphor; BP1, benzophenone-1; BP2, benzophenone-2; 4HB, 4-hydroxybenzophenone; 4DHB, 4,4'-dihydroxybenzophenone; THB, 2,4,4'-trihydroxybenzophenone; BP3, benzophenone-3; BP4, benzophenone-4; PBS, 2-phenly-5-benzimidazole-sulfonic-acid; IMC, isopentyl-4-methoxycinnamate; OMC, octyl-methoxycinnamate; OC, octocrylene; BS, benzylsalicylate; PS, phenlysalicylate; HMS, homosalate; OS, octyl salicylate; PABA, 4-aminobenzoic acid; PEG25-PABA, ethoxylated ethyl-4-aminobenzoate; OD-PABA, octyl dimethyl PABA; BIM, bisimidazylate; BM-DBM, 4-tert-Butyl-4'-methoxydibenzoylmethane; ECL, 2,2-methylenbis-phenol; UAB, Uvinul A plus B.

yeast extract). Thus prior to the assay yeast cell growth was conducted as described (Petit *et al.* 1995), but with the modification that yeast colonies from CM medium were inoculated in Erlenmeyer flasks containing 15 mL of YPD growth medium instead of CM medium, which lead to increasing growth rates and better assay performance.

rtERα assay procedure. The whole assay was performed as described in detail previously (Le Guével and Pakdel 2001; Petit *et al.* 1995) but instead of hemolysis tubes in clear polystyrene 96-well microplates (Greiner Bio-One, Huber AG, Basel, Switzerland) were used leading to small modifications of the assay procedure according to (Schultis and Metzger 2004). Thereby the centrifugation step after cell lysis was excluded and the lysed suspension was transferred to the flat bottom 96-well plate, instead of the supernatants only. The protein measure (Petit *et al.* 1995) was replaced by measuring yeast turbidity (A_{620}), in order to assess and correct for yeast growth and as a control for cytotoxicity. Cytotoxicity was maniftested by significantly reduced yeast growth or even cell lysis and was determined by absorbance at 620 nm. High concentrations of some UV filters that lead to cytotoxicity were omitted from curve fitting and calculations.

For screening of the UV filters a 96-well V-bottomed microtitre plate was filled with 100 µL/well *S. cerevisiae* cells in YPD culture. Three rows contained serially diluted positive control E2, one row the ethanol-blank, and four rows the analysed UV filter in quadruplicates with increasing concentrations resulting in dose response curves. After cell lyses the lysed suspension was transferred to a new flat-bottom 96-well plate (Greiner Bio-One, Huber AG, Basel, Switzerland), ONPG was added and the estrogenic activity measured as previously described (Le Guével and Pakdel 2001; Petit *et al.* 1995; Schultis and Metzger 2004).

The hERα recombinant yeast was kindly provided by J. Sumpter, Brunel University and the assay was performed according to Routledge and Sumpter (1996) and Kunz and Fent (submitted). The yeast (*S. cerevisiae*) genome carries a stably integrated DNA sequence of the human estrogen receptor (hERα), and they also contain expression plasmids carrying estrogen responsive elements, regulating the expression of the reporter gene lacZ (encoding the enzyme β-galactosidase). Thus, when an active ligand (i.e. E2 or an estrogenic UV filter) binds to the receptor, β-galactosidase is synthesised and secreted into the medium, leading to a colour change of chromogenic substrate chlorophenol red ß-D-galactopyranoside (CPRG) from yellow to red.

Experiments in vivo in fish

Fish. The 14-days fish experiments were conducted using juvenile, sexually undifferentiated fathead minnows (*Pimephales promelas*), between 2-3 months of age and with a total body length between 19-27 mm. This fish species has been chosen because of its frequent used in the field of endocrine disrupters and established techniques including vitellogenin (VTG) antibodies. The experimental procedure and duration was similar to that of Panter *et al.* (2002)**,** who showed that estrogens and antiestrogens are detectable after 14 days of exposure by virtue of the VTG response.

Mixed sex juvenile fathead minnows were received from the cultivator (Aquatic Research Organisms, Hampton NH, USA) and adapted for a minimum of 14 d in our laboratory in aquaria prior to the experiment. Fish were fed with Tetramin pellets (Tetra GmbH, Melle, Germany) twice a day with a quantity of 1% of body weight prior to the onset of experiments. During the experiments, fish were fed with brimeshrimp (*Artemia salina*, Argent Chemical Labs, Redmond WA, USA) at a feeding rate of 1% of body weight twice a day.

Exposure. Fish were held in well-aerated reconstituted tab water medium (total hardness 160 mg/L as $CaCO_3$, total alkalinity 30 mg/L as $CaCO_{3,}$ conductivity 500 µs/cm) and a 16 h/8 h light/dark cycle at 25±1°C. The studies were conducted using a 24 h static-renewal procedure with daily renewal of total aquaria water. For exposure, 10 randomly selected fish each were placed in stainless steel tanks (10 L) and exposed to individual UV filters for 14 d. Not all UV filters evaluated *in vitro* could be analysed *in vivo*. In order to have an reasonable amount of *in vivo* experiments, UV filters were selected as follows: either because they exhibited maximal estrogenic activities in our *in vitro* assays (BP1, BP2, 4DHB), or because they possessed submaximal (BP3, BP4, 3BC) or no (4MBC, OMC) estrogenic activities in our *in vitro* assays, but were reported to be estrogenic by other studies and because of its frequent use.

First, an experiment was performed with 4MBC, 3BC, BP1 and BP2, and second, an experiment with BP3, BP4, OMC and 4DHB. In both experiments a control, solvent control (SC, 1 mL ethanol in 10 L water) and positive control for estrogenic activity (100 ng/L E2) were included. Stock solutions of each chemical were prepared freshly in ethanol prior to the start of the experiment and added daily to the experimental water by mixing. The following nominal concentrations of UV filters were used: 10, 100, 500, 1'000 and 5'000 µg/L for BP1, BP3, BP4, OMC and 4DHB, respectively; 10, 100, 500 and 1'000 µg/L for 4MBC and 3BC, respectively; and 10, 100, 500, 1'000 and 10'000 µg/L for BP2.

The concentrations were selected on the basis of environmental residues and including higher levels in order to span a large concentration range. Toxic side effects (i.e. lethargy, uncoordinated swimming, loss of equilibrium, hyperventilation) were observed for fish exposed to 5'000 µg/L BP3 and 1'000 µg/L 4MBC, and these experiments were stopped at day 8 of exposure.

Physico-chemical measurements and biological observations. Physico-chemical para-meters were determined daily. pH and oxygen saturation ranged between 7.2–7.9 and 6.5–8.3 mg/L, respectively, throughout the whole exposure period. Mortalities and abnormal behavior were recorded daily and dead fish were removed from the tanks. On day 14 all fish were anaesthesized with buffered tricaine methan sulfonate (MS-222, 100 mg/L with 200 mg $NaHCO_3$/L). Subsequently individual fish were measured, weighted, transferred into labelled Eppendorf® tubes, frozen and stored at -20 °C for homogenisation and VTG analysis.

Vitellogenin analysis. Fish were defrosted at 4°C and individually homogenized in ice-cold assay buffer (Biosense, Bergen, Norway) in a 1:2 ratio wet weight:buffer volume, using a Ultra Turax homogenizer (IKA, Huber + Co. AG, Reinach, Switzerland). The homogenates were centrifuged at 10'000 g for 3 min at room temperature using a microcentrifuge (Eppendorf centrifuge 5415 D, Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). The supernatant was withdrawn and immediately used for vitellogenin (VTG) analysis or frozen at –80 °C until required for VTG analysis. Whole-body homogenates were assayed for VTG using a quantitative heterologous carp enzyme-linked immunosorbent assay, which has been shown to be highly reliable for VTG determination in fathead minnow (Panter *et al.* 2002; Tyler *et al.* 1999). The commercially available quantitative carp vitellogenin ELISA kit, which is based on a sandwich ELISA format (Biosense, Bergen, Norway), was used for determination of VTG in whole body homogenates of individual fish and was conducted as described by Biosense. Purified carp VTG from blood plasma (Biosense, Bergen Norway) was used as a standard for quantitation according to the provider's description.

Analytical chemistry. For the duration of the experiment four aliquots of 250 mL exposure waters were taken from the two highest and the two lowest concentrations of each UV filter and controls at the beginning (0 h) and prior to water renewal (24 h). The aliquots of the same concentration of UV filter were pooled for each UV filter at each concentration and time point in brown glass flasks, preserved by acidification using HCl to pH 2-3 and stored at 4°C until analysis. Chemical analyses of UV filter concentrations were carried out by high performance liquid chromatography and UV detection. Briefly, 25 or 250 mL of water samples, depending on sample concentration, were extracted and concentrated by solid phase extraction (SPE). The 2'500-times concentrated eluent was then analyzed by HPLC-DAD.

Data processing and statistical analysis

Recombinant yeast assay. The absorbance measurement at 405 nm (ONPG) and 620 nm (turbidity) for the rtERα assay allowed for subsequent correction for turbidity (yeast growth) as follows:

$$\text{Corrected absorbance} = \text{chemical absorbance}_{405\,\mathbf{m}} - \text{chemical absorbance}_{620\,\mathbf{m}} - [\text{blank absorbance}_{620\,\mathbf{m}} - \text{blank absorbance}_{405\,\mathbf{m}}]$$

For all UV filters the maximal response relative to the standard (=100%) were calculated. Thereby the height of the UV filter dose-response curve was expressed as percentage of the maximal effect produced by the dose response curve of E2.
High concentrations of some UV filters that inhibited growth of the yeast, or even lysed cells were omitted from curve fitting and calculations. For curve fitting and EC50 calculations (GraphPad Software Inc., San Diego, USA), the corrected absorbance values versus the logarithm of concentration were plotted, whereby the best fit from a number of non-linear regression models was selected for final data analysis. In this study, we used the Hill equation (or sigmoidal dose-response with variable slope) to fit full dose-response curves, which reached the same height (≥ 80% maximal response) as the corresponding standard E2. Moderate (30-80% maximal response) and submaximal (< 30% maximal response) dose-response curves were fitted using the best fit from a number of non-linear regression models. Coefficient of determination (R^2), residuals and 95% confidence intervals were calculated and the runs test was carried out to verify that the fitted curve represents data correctly. Estrogenic potencies were calculated for all active UV filters. Thereby the EC50 of UV filters with full dose-response curves was divided by the EC50 of the E2 standard. For UV filters with submaximal dose-response curves, estrogenic potencies were estimated based on their EC50 values, despite differences in curve-steepness and –height when compared to the standards. Thereby a good approximation of the estrogenic potencies of submaximal UV filters was achieved.

Fish experiment. After testing the data distribution for normality by using the Kolmogorov-Smirnov test, means of wet weight and total length of individual fish were calculated and data analysed by analysis of variance (ANOVA) followed by a Dunnett's Multiple Comparison test to compare the treatment means with respective controls. Means of VTG concentrations of individual fish were calculated and data analysed with the non-parametric Kruskal-Wallis test followed by a Dunn's Multiple Comparison test to compare the treatment means with respective controls. Statistical comparisons with the control were made using the SC as the overall control. The results are given as mean ± standard error of mean (SEM). Differences were considered significant at $p \leq 0.05$. All computations were performed with PRISM 4.0 (GraphPad Software Inc., San Diego, USA).

Results

Estrogenic activity of UV filters in vitro

Ten of 23 analyzed UV filters and the UV filter metabolite 4HB were found to possess estrogenic activity in the recombinant yeast assay expressing the rainbow trout ERα. BP1, BP2, 4DHB, THB, 4HB and PS were full rtERα agonists exhibiting full dose response curves. They had maximal responses of 81 to 123% as compared to E2 (Fig. 1). Moderate, but clear dose-response curves were found for BS, BP3 and BP4, characterized by lower maximal responses of 43-74% (Fig. 1). Submaximal dose response curves were observed with 3BC and OS with maximal responses of 27% and 15%, respectively (Fig 1). Table 2 shows the relative potencies of the UV filters compared to E2, as determined by their half-maximal induction activities (EC50). The most potent UV filter was BP1, which was only 87-times less potent then E2. Estrogenicity decreased in the following order 4HB > 3BC > BS > BP2 > BP3 > THB > PS > 4DHB > BP4 > OS. The estrogenic potencies were in the range of 390 to 24'750 times lower than E2. The remaining 12 UV filters, namely 4MBC, BIM, BM-DBM, ECL, HMS, IMC, OC, PABA, OD-PABA, PEG25-PABA, OMC, PBS and UAB were inactive up to $2.5x10^{-2}$ M. In every assay we checked for potential cytotoxicity caused by the UV filter, by routinely measuring yeast growth (620 nm) besides β-galactosidase activity (405 nm). Hence UV filter concentrations, which lead to reduced yeast cell growth or complete growth inhibition, were omitted from data analysis for hormonal activities. At high concentrations slight cytotoxicity occurred for BS ($\geq 5.04 \times 10^{-5}$ M), BP4 ($\geq 10.0 \times 10^{-4}$ M), 4DHB ($\geq 1.00 \times 10^{-3}$ M) and PS ($\geq 1.00 \times 10^{-4}$ M). Hormonal activity was therefore assessed at non-cytotoxic concentrations only.

Comparison with hERα. The same UV filters found active in our present study with recombinant yeast expressing the rainbow trout ERα were previously found active in a recombinant yeast system expressing the human estrogen receptor alpha (hERα). By using this system we investigated 17 UV filters and one metabolite for their multiple hormonal activities such as estrogenicity, antiestrogenicity, androgenicity and antiandrogenicity *in vitro* (Kunz and Fent, submitted). The only exception was OS that exhibited minimal estrogenic activity in the rtERα only. The activities of BP1, 3BC and the salicylates were relatively higher with rtERα than hERα, but lower for the remaining benzophenones displaying lower EC50 values with hERα. BP1 and 4HB showed strongest activities in both receptor systems. The rankings of the other UV filters differed between the receptor systems, however. Whereas in the hERα assay benzophenone derivates were the most potent compounds benzophenone-, camphor-, and salizylate-derivates were most potent in the rtERα assay. The maximal responses of estrogenic compounds in the rtERα assay were in most cases higher than in the hERα, with only BP2 and 4DHB as exceptions (Table 2). A direct comparison of both assays is shown in Figure 2. BP1 as most potent UV filter in both assays displayed an estrogenic activity of only 87-times less

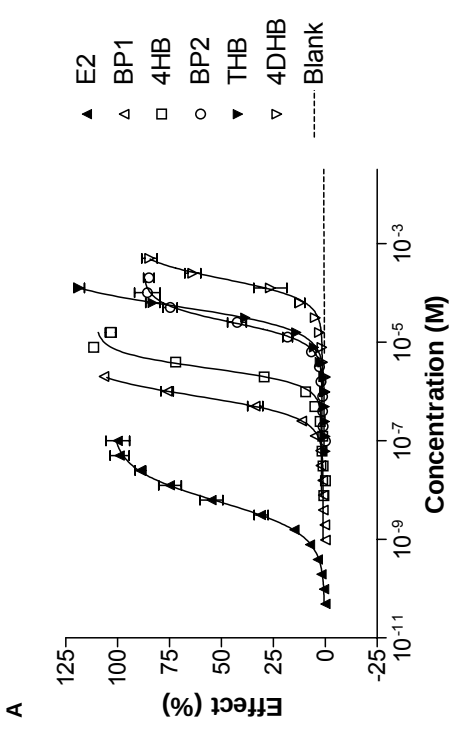


Figure 1 Estrogenic activity of UV filters in the rtERα assay, shown in two panels (A, B) for clarity. Data shown are means ± SEM (3 experiments with 4 replicates each). Effect (%) represents percentage of β-galactosidase induction of UV filters versus solvent (0%) and E2 (100%) controls. Results for inactive chemicals are not shown for clarity. Compound abbreviations see Table 1.

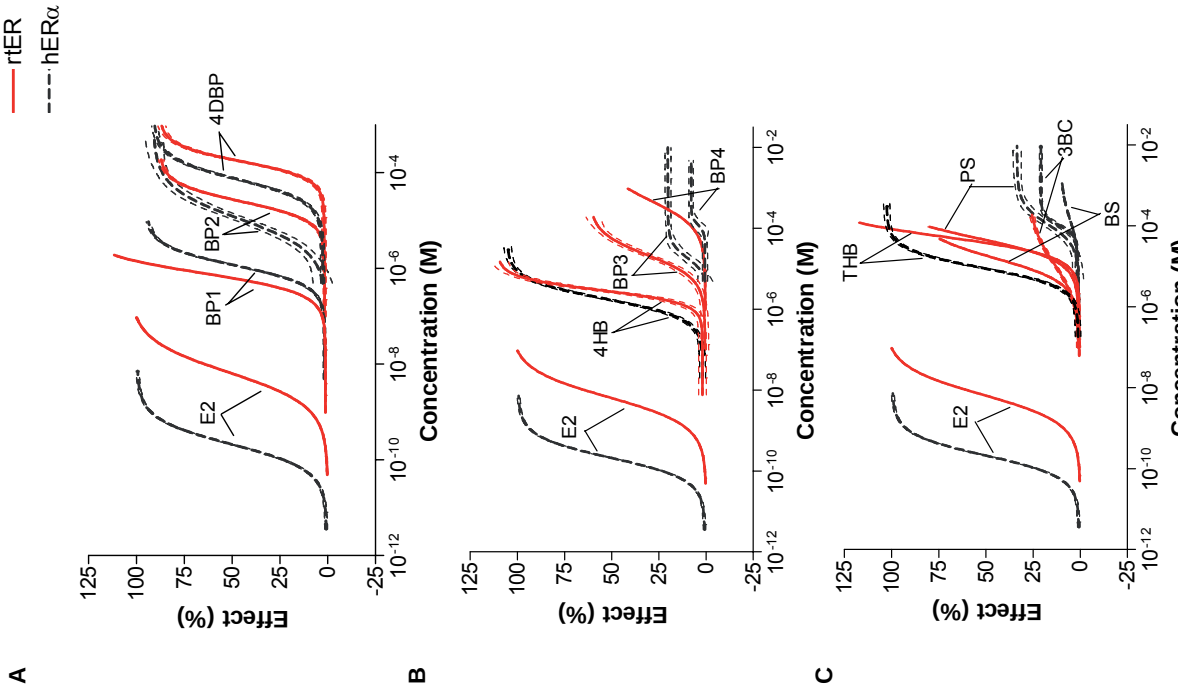


Figure 2 Comparison of estrogenic activity of UV filters between the rtERα (bold lines) and the hERα (dashed lines) assay, shown in three panels (A, B, C) for clarity. Data shown are means and 95% confidence intervals (3 experiments with 4 replicates each). Effect (%) represents percentage of β-galactosidase induction of UV filters versus solvent (0%) and E2 (100%) controls. Results for inactive chemicals are not shown for clarity. Compound abbreviations see Table 1.

than that of E2 with rtERα and 5'000-times less with hERα. In particular, the relative activity of 3BC was higher in the rtERα assay. In contrast to the relatively higher activity of UV filters in the rtERα assay, the hERα assay was 62-times more sensitive towards E2

Estrogenic activity of UV filters in fish in vivo

Measured exposure concentrations. Concentrations of UV filters in aquaria waters were measured at the beginning of exposures (0 h) and 24 h later prior to water renewal at the lowest and the two highest exposure concentrations in order to determine actual effect concentrations and to get an estimate of concentration decrease. Concentrations decreased during the exposure, but to a variable extent for different compounds. Table 3 shows that actual concentrations determined by HPLC analysis were close to nominal. After 24 h before water renewal, concentrations decreased to various degrees (0-32%) depending on compound and concentration. The different concentration decreases are a result of different physico-chemical properties of UV filters (lipophilicity) and uptake by fish.

Effects of UV filters on fish survival, weight and length. No mortality was observed in controls, solvent controls (SC) and positive controls (E2) exposed fish in both experiments. The UV

Table 4. Body weight and length of exposed fish after 0 and 14 days of exposure.

	Exposure (μg/L)	Body weight (mg)	Body length (mm)
Controls	Day 0	62.0 ± 24.4	18.9 ± 2.2
	Water	211.3 ± 91.1	29.4 ± 2.8
	Solvent	238.0 ± 88.8	30.8 ± 3.5
	E2	249.0 ± 111.8	29.6 ± 4.1
4MBC	9	272.4 ± 82.9	31.1 ± 2.9
	100	237.3 ± 60.6	30.4 ± 2.4
	415	103.9 ± 40.8 **	23.6 ± 2.6 **
	753	43.1 ± 25.2 **	17.1 ± 3.6 **
3BC	9	335.9 ± 128.9 *	32.8 ± 3.1
	100	258.9 ± 95.9	30.9 ± 3.4
	435	115.1 ± 37.4 **	24.7 ± 2.4 **
	953	95.8 ± 25.8 **	23.7 ± 1.8 **
BP1	9	246.5 ± 77.0	30.0 ± 2.6
	100	273.1 ± 141.2	31.7 ± 4.6
	500	240.4 ± 67.8	30.5 ± 2.1
	981	245.0 ± 90.0	29.4 ± 3.8
	4'919	121.9 ± 46.9 *	24.6 ± 3.2 **
BP2	10	272.9 ± 125.7	30.9 ± 4.9
	100	264.5 ± 116.9	30.6 ± 4.1
	500	247.6 ± 73.7	30.5 ± 2.2
	1'067	293.4 ± 113.4	32.1 ± 3.6
	8'783	171.4 ± 35.0	25.9 ± 1.8 **

	Exposure (μg/L)	Body weight (mg)	Body length (mm)
Controls	Day 0	161.3 ± 45.7	27.4 ± 2.1
	Water	313.1 ± 80.0	33.8 ± 3.1
	Solvent	277.0 ± 62.2	32.0 ± 2.2
	E2	282.9 ± 70.7	32.4 ± 2.6
BP3	12	313.9 ± 59.9	34.6 ± 2.0
	100	317.5 ± 05.4	33.3 ± 3.7
	500	281.6 ± 23.7	32.7 ± 3.5
	766	257.3 ± 51.1	32.0 ± 1.7
	3'900	134.6 ± 36.6 **	27.9 ± 2.2 *
BP4	11	330.5 ± 14.6	34.2 ± 4.4
	100	326.4 ± 02.2	33.4 ± 3.0
	500	309.2 ± 69.3	34.1 ± 1.9
	1'048	299.9 ± 57.8	33.5 ± 1.7
	4'897	375.4 ± 97.5 *	35.5 ± 2.8 *
OMC	8	303.3 ± 14.5	33.4 ± 3.6
	100	266.7 ± 54.5	32.4 ± 2.5
	500	283.3 ± 62.6	32.8 ± 1.8
	889	303.2 ± 65.8	33.2 ± 2.2
	5'025	267.4 ± 60.5	32.4 ± 2.1
4DHB	10	321.8 ± 70.2	34.7 ± 1.1
	100	316.9 ± 11.4	34.0 ± 3.4
	500	340.9 ± 02.2	34.4 ± 3.0
	900	351.4 ± 93.7	34.5 ± 2.6
	5'011	371.5 ± 97.0	35.5 ± 3.0 *

E2: 17β-Estradiol; **, Significantly different from solvent control at $p < 0.01$; *, Significantly different from solvent control at $p < 0.05$.

Table 2. Comparison of *in vitro* (rtERα, hERα) and *in vivo* effect concentrations.

	In vivo		In vitro								
	Vtg. Induction		rtER assay EC50		Maximal response	Potency (1/...)	hER assay EC50		Maximal response	Potency (1/...)	Sensitivity Ratio EC50
	(µg/L)	(M)	(µg/L)	(M)			(µg/L)	(M)			(rtER/hER)
E2	0.1	$3.67x10^{-10}$	4.93	$1.81x10^{-8} \pm 5.12x10^{-9}$	100%	1	0.08	$2.91x10^{-10} \pm 1.19x10^{-10}$	100%	1	62
4MBC	*n.e.*		*n.e.*				*n.e.*				--
3BC	953	$3.96x10^{-6}$	2'927	$1.22x10^{-5}$	27%	960	74'443	$3.10x10^{-4}$	21%	$1.3 x10^{6}$	0.04
	435	$1.81x10^{-6}$									
BP1	4'919	$2.30x10^{-5}$	171.26	$7.99x10^{-7}$	114%	87	247.15	$1.15x10^{-6}$	96%	5'000	0.7
BP2	8'783	$3.57x10^{-5}$	6'141	$2.49x10^{-5}$	88%	2'690	2'684	$1.09x10^{-5}$	91%	21'000	2.3
4DHB	*n.e.*		36'867	$1.72x10^{-4}$	88%	23'340	15'727	$7.34x10^{-5}$	91%	170'000	2.3
4HB	--		586.73	$2.96x10^{-6}$	111%	390	360.56	$1.82x10^{-6}$	108%	16'000	1.6
THB	--		10'506	$5.30x10^{-5}$	123%	7'890	1'818	$9.17x10^{-6}$	103%	27'730	5.8
BP3	*n.e.*		4'999	$2.19x10^{-5}$	62%	3'470	4'237	$1.86x10^{-5}$	18%	45'000	1.2
BP4	*n.e.*		91'846	$2.98x10^{-4}$	43%	24'750	29'241	$9.48x10^{-5}$	6%	380'000	3.1
IMC	--		*n.e.*				*n.e.*				--
OMC	*n.e.*		*n.e.*				*n.e.*				--
OC	--		*n.e.*				*n.e.*				--
BS	--		2'614	$1.15x10^{-5}$	74%	1'800	37'872	$1.66x10^{-4}$	12%	860'000	0.07
PS	--		6'704	$3.13x10^{-5}$	81%	8'200	23'648	$1.10x10^{-4}$	32%	480'000	0.3
HMS	--		*n.e.*				*n.e.*				--
OS	--		964'772	$3.85x10^{-5}$	15%	570'000	*n.e.*				--
PABA	--		*n.e.*				*n.e.*				--
PEG25 PABA	--		*n.e.*				*n.e.*				--
OD-PABA	--		*n.e.*				*n.e.*				--
BIM	--		*n.e.*				*n.e.*				--
BM-DBM	--		*n.e.*				*n.e.*				--
ECL	--		*n.e.*				*n.e.*				--
UAB	--		*n.e.*				*n.e.*				--

Abbreviations: E2, 17β-estradiol; EC_{50}, the concentration of the compound exhibiting 50% of its total effect. n.e., no effect. Values for E2-standard are given in mean S.E.M. of 10 (rtERα) or 9 (hERα, Kunz et al submitted) experiments, respectively with three replicates each. EC_{50} values of compounds are from 3 experiments with four replicates each. Potency = EC_{50} compound/EC_{50} E2 is calculated from mean values of each single experiment; the used EC_{50} values for E2 in each experiment are not listed here for simplicity reasons. Chemical concentrations shown for the *in vivo* assay are those at which VTG was significantly induced relative to the solvent control. For compound abbreviations see table 1.

Table 3. Nominal and measured water concentrations of analyzed UV filters.

	Exposure concentrations				
	Nominal	Measured			
	(µg/L)	0 h (µg/L)	24 h (µg/L)	Median (µg/L)*	After 24 h (%)**
3BC	10	9.5 ± 0.3	8.0 ± 1.2	9	**84%**
	500	516.7 ± 36.7	352.5 ± 130.8	435	**68%**
	1'000	1'070.0 ± 28.3	835.0 ± 49.5	953	**78%**
BP1	10	9.8 ± 0.1	8.1 ± 0.2	9	**82%**
	1'000	1'032.4 ± 54.8	930.0 ± 14.1	981	**90%**
	5'000	5'191.7 ± 58.7	4'647.1 ± 221.4	4'919	**90%**
BP2	10	10.7 ± 0.6	9.9 ± 0.9	10	**93%**
	1'000	1'102.5 ± 9.8	1'031.7 ± 17.0	1'067	**94%**
	10'000	9'747.3 ± 557.9	7'818.5 ± 104.5	8'782	**80%**
4MBC	10	9.6 ± 1.8	7.4 ± 0.4	9	**77%**
	500	492.4 ± 102.7	337.5 ± 17.7	415	**69%**
	1'000	826.1 ± 189.4	680.0 ± 198.0	753	**82%**
OMC	10	8.8 ± 0.1	6.5 ± 0.7	8	**74%**
	1'000	1'012.5 ± 165.6	765.0 ± 63.6	889	**76%**
	5'000	5'450.0 ± 282.8	4'600.0 ± 141.4	5'025	**84%**
BP3	10	13.5 ± 1.1	9.7 ± 0.2	12	**71%**
	1'000	879.5 ± 115.0	652.2 ± 65.4	766	**74%**
	5'000	4'175.0 ± 247.5	3'625.0 ± 106.1	3'900	**87%**
BP4	10	11.5 ± 0.9	10.8 ± 0.8	11	**94%**
	1'000	1'068.2 ± 64.4	1'027.7 ± 99.2	1'048	**96%**
	5'000	5'158.9 ± 425.2	4'634.3 ± 26.4	4'897	**90%**
4DHB	10	11.8 ± 1.0	9.0 ± 0.1	10	**76%**
	1'000	901.9 ± 48.4	899.0 ± 42.9	900	**100%**
	5'000	5'388.5 ± 2.7	4'633.0 ± 232.4	5'011	**86%**

*: Median of actual concentrations at 0 h and 24h
**: Percentage of actual concentration at 24 h relative to 0h

filters did not affect survival during exposure, except at the highest concentrations of 4MBC, 3BC and BP1. After 8 d of exposure, two fish died at 753 µg/L 4MBC (survival 80%) and the experiment was stopped. At 953 µg/L 3BC and 4919 µg/L BP1, one fish each was found dead at day 12 and 11, respectively. Fish at 8783 µg/L BP2 showed some signs of edema at the end of exposure.

During the 14 d exposures, all control, SC and E2 fish grew as determined by increase in wet weight and total body length (Table 4). At low concentrations of UV filters no significant differences to controls was observed. Length gain was significantly decreased for 500 and 1000 µg/L 3BC and 4MBC (Table 4), however. No difference occurred in wet weight and mean length in the SC and E2. 3BC and 4MBC led to a dose related decrease in the weight gain and body length at 435 and 953 µg/L, and 415 and 753 µg/L, respectively. No decreases in body weight gain and body length were observed with all other UV filters at all exposure concentrations, except for 4919 µg/L BP1 after 14 d of exposure.

Estrogenicity of UV filters. Significant VTG induction occurred in fish exposed to the 100 ng/L E2. Mean whole-body VTG content was 2'600 µg/mL and highly induced, compared to the water and solvent control having a residual level of 0.3 µg/mL. Dose-dependent increases in VTG were observed in fish exposed to 3BC, BP1 and BP2 (Figure 3). 3BC showed higher VTG induction and at lower concentration compared to BP1 and BP2. Dose-related significant

VTG induction occurred at 3BC concentrations of 435 µg/L (407 µg VTG/mL) and 953 µg/L (1753 µg VTG/mL). Concentration related VTG induction was also found after exposures to higher concentrations of BP1 and BP2. Although increased at medium concentrations, VTG induction was significant only at

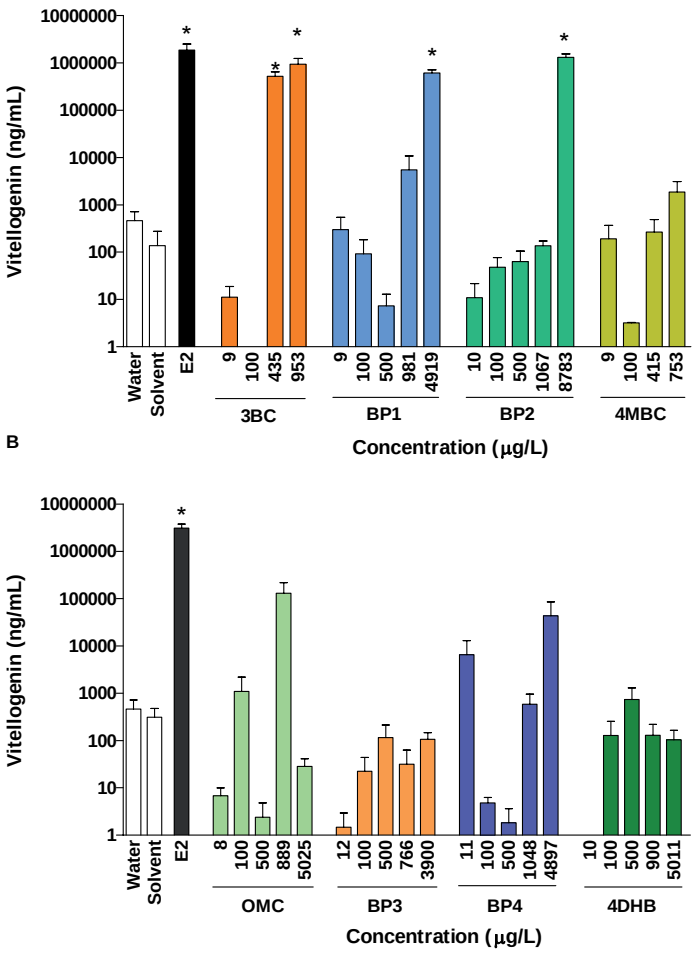


Figure 3 Vitellogenin concentration in juvenile fathead minnows exposed to eight UV filters. Values are means ± SEM (n=10). Asteric denotes a significant difference from control (solvent) at $p \leq 0.05$. Concentrations given as actual median measured, except 100 and 500 µg/L.

the highest concentrations of BP1 and BP2, namely at 4919 µg/L BP-1 (907 µg VTG/mL), and 8783 µg/L BP2 (1504 µg VTG/mL). The UV filters BP3, BP4 and 4DHB did not result in a significant VTG induction at all exposure concentrations in fish, although they showed submaximal estrogenic activity *in vitro*. 4MBC and OMC, which showed no estrogenicity *in vitro*, were not estrogenic *in vivo*. Therefore, two of five UV filters which exhibited estrogenic activity *in vitro* were also estrogenic *in vivo*.

Discussion

In our present study we show that it is most appropriate to determine the endocrine-disrupting activity of chemicals both *in vitro* and *in vivo*, preferably in a tiered approach, as no single assay may be best suited to determine the hormonal activity of a compound and because of species differences. Thereby we demonstrate for the first time that as many as ten of 23 commonly used UV filters are estrogenic in an *in vitro* yeast assay carrying a fish ER (rtER). Compared to our results in the recombinant yeast carrying the hERα, where we investigated 17 UV filters and one metabolite for estrogenicity, antiestrogenicity, androgenicity and antiandrogenicity *in vitro* (Kunz and Fent submitted), we found that all compounds, except OS, were equally estrogenic in both assays despite lower activity of E2 in the rtERα assay. In fish we demonstrated that three of nine UV filers were estrogenic *in vivo*. Comparing *in vitro* activities in two systems with fish *in vivo* activity, we found the rtERα *in vitro* data more accurate than the hERα data for prediction of the *in vivo* activity. Hence estrogenic activity of chemicals is best assessed by the use of a tiered approach with a combination of *in vitro* and *in vivo* assays of the same species.

In vitro activity in the rtERα assay. We found ten of 23 compound exhibiting estrogenic activities in the rtERα assay with different maximal responses and dose response curves. The range of moderate to full dose-response curves can be explained by the molecular structures. UV filters displaying full dose-response curves are characterized by at least one ring substituted hydroxyl group. They display lower maximal responses with increasing molecular symmetry. Additional substituents on the phenolic ring have a diminishing effect on the maximal estrogenic responses in both ERα systems (Kunz and Fent submitted; Routledge and Sumpter 1997). This is the case for BP3, BP4, BS, OS and 3BC, which are substituted with methoxy-, sulfonic acid-, benzyl, octyl or camphor groups, possibly indicating only partial agonism. Inactive UV filters had with very few exceptions, like those compounds with very large molecular structures that prevent uptake into cells, only one non-hydroxylated ring that was connected and/or attached to other substituents such as ethoxy, alkyl-, amino-, cyano- or methoxy-groups, which were shown to significantly decrease the chemical's affinity for the rtERα, as previously shown for hERα (Blair *et al.* 2000). The structural basis of the estrogenic UV filters found in our study goes in

line with recent findings on structure activity relationships of structurally similar chemicals in hER-systems (Miller *et al.* 2001; Routledge and Sumpter 1997; Schultz *et al.* 2000).

Comparison between rainbow trout and human ERα. We were interested in elucidating whether the structural differences between human and rainbow trout ERα (Petit *et al.* 1995; Petit *et al.* 2000) were responsible for functional differences. Homologies in amino acid sequences between hERα and rtERα are variable, depending to the domains (Pakdel *et al.* 1990). The most highly conserved region is the C domain (92% homology), which is responsible for DNA-binding and dimerization (Petit *et al.* 2000). Whereas rtERα and hERα have similar binding affinities to an estrogen response element, the rtERα C domain is responsible for a weaker DNA binding stability. The E domain shares 60% similarity between rtERα and hERα and contains the hormone binding domain. Petit *et al.* (1995) found that the rtERα has a lower affinity to E2 than the hERα. This was further demonstrated for 17α-estradiol, estrone and 17α-ethinylestradiol (Le Guével and Pakdel 2001) and is confirmed by our present study; the E2 concentration necessary to induce 50% activity was 62-times higher in the rtERα assay. The weaker magnitude of E2 stimulation mediated by rtERα is attributed to the lower DNA-binding stability, rather than structural differences of the two ER (Petit *et al.* 2000).

As for most of the UV filters, relative sensitivities of rtERα and hERα systems varied only little (one order of magnitude), indicating that the main difference between the two receptors is their sensitivity towards E2 (Table 2). Differences between the two ER occurred for the salicylate derivatives (PS, BS, OS), which showed several times higher activity in rtERα, and 3BC that showed even 1'300-times higher activity than in the hERα. Maximal responses were generally higher in the rtERα assay, except for BP2 and 4DHB. This indicates a higher relative sensitivity and weaker partial agonism in the rtERα of compounds showing submaximal activity.

Thus in contrast to the lower activity of E2, the activity of some UV filters is relatively higher in the rtERα assay. This cannot be fully explained by a lower DNA-binding stability found by Petit *et al.* (2000) for estradiol, but is rather attributed to structural differences of the two ER and the molecular structure of the UV filters interacting with the ER. This points toward a slightly different substrate binding specificity of the fish and human ERα based on differences in the binding domain of the two receptors. In addition, differences in the transactivation process such as dimerization and DNA-binding capacity may also account in part for the different relative sensitivities. Forthcoming studies focusing on UV filter receptor binding and influence on the transactivation process will elucidate the reasons for the differing sensitivities of the fish and hERα.

Comparison with other in vitro studies. The estrogenicity of UV filters found in our study with rtERα is consistent with results obtained *in vitro* in human ER-systems, although relative

sensitivities may differ. Estrogenicity of some salicylate and camphor derivatives have been reported in mammalian systems such as recombinant yeast (Kunz and Fent submitted; Miller *et al.* 2001; Mueller *et al.* 2003), receptor binding assays (Blair *et al.* 2000; Mueller *et al.* 2003; Schlumpf *et al.* 2004), proliferation of MCF-7 cells (Schlumpf *et al.* 2001; Schlumpf *et al.* 2004) and reporter gene induction in transfected cell lines (Schreurs *et al.* 2002; Suzuki *et al.* 2005; Yamasaki *et al.* 2003). The estrogenicity of BP1, BP2, BP3, 3BC, found in our rtERα assay is consistent with findings in MCF-7 cells (Schlumpf *et al.* 2001; Schlumpf *et al.* 2004), reporter hERα/HeLa cells (Yamasaki *et al.* 2003), MCF7 reporter cells (Suzuki *et al.* 2005) and HEK293 cells (Schreurs *et al.* 2005). Our results are also consistent with the hERα cell assay for BP3, but not for 4MBC in the HEK 293 reporter gene assay. In addition, 3BC, HMS and 4MBC showed activity in the hERα assay (Schreurs *et al.* 2002).

In fish at least two ER subtypes, ERα and ERβ, occur, and in zebrafish, a third form has been reported (Menuet *et al.* 2002). Currently it is not known to what extent UV filters interact with these receptors. Reasons for differences between results obtained in our study with the rtERα assay and other *in vitro* assays are first, that UV filters may be active towards the ERβ, but not the ERα. In the MCF-7 and other human cells, active UV filters may interfere with both hERs. 4MBC was estrogenic in the MCF-7 cells (Schlumpf *et al.* 2001), but did not exhibit estrogenic activity towards the rtERα in our present study, similarly as previously reported from our experiments with the hERα (Kunz and Fent submitted), which is based on the fact that 4MBC binds preferably to the ERβ (Schlumpf *et al.* 2004). This is also the case for HMS (Schreurs *et al.* 2002). In addition yeast has only a low capability for metabolism, therefore metabolites of UV filters binding to the ER are not identified by the rtERα assay. The differences may also partly depend on different binding activities of the ERs of different species, and between *in vitro* assays and their different capabilities to metabolically activate a chemical. This leads to the conclusion that species differences in the estrogenic activity occur and that one *in vitro* assay alone is not sufficient enough for assessing the estrogenicity of chemicals for fully characterizing its estrogenic potential. Moreover homologuous *in vitro* systems are more reliable for predicting *in vivo* activity.

Comparison of in vivo activities. Our *in vivo* experiments demonstrate that of eight analysed UV filters, 3BC, BP1 and BP2 showed estrogenic activity in fathead minnows. 3BC led to dose dependent induction of VTG at lower concentrations (435, 953 µg/L) than BP1 (4919 µg/L) and BP2 (8783 µg/L). 4MBC, OMC, BP3, BP4 and 4DHB did not induce VTG up to the highest concentrations in the range between 753 µg/L (4MBC) and 5010 µg/L (4DHB). Schreurs *et al.* (2002) observed no estrogenicity in transgenic zebrafish exposed for 96 h at 10 µM of OMC (2.90 mg/L), OD-PABA (2.77 mg/L), HMS (2.62 mg/L), BP3 (2.28 mg/L), and 1 µM of 4MBC (0.25 mg/L), which is consistent with our data with these UV filters analysed at similar concentrations, but for a longer period of time. Injection of high concentrations of

3BC (27, 68, 137 mg/kg and higher) induced VTG in rainbow trout (Holbech *et al.* 2002). In medaka, estrogenic activity of 4MBC and OMC was observed only at about 200-times higher concentrations (Inui *et al.* 2003), but was not found at 20-times higher concentrations than in our study. This may be related to species-specific differences in VTG induction. The relative degree of VTG induction is species-specific as shown for rainbow trout that reacted with higher VTG induction to endocrine disrupters than roach (Routledge *et al.* 1998).

The UV filters 4MBC, BP3, 4DHB and OMC exhibited estrogenicity *in vivo* in rats (Mueller *et al.* 2003; Schlumpf *et al.* 2001; Schlumpf *et al.* 2004; Yamasaki *et al.* 2003), but this was not observed in our fish study. The differences can be explained by species differences in metabolism and different affinities to the ERs, as 4MBC and HMS preferably bind to the ERβ (Schlumpf *et al.* 2004; Schreurs *et al.* 2002). Whether this is the case in fish is currently not known. Most likely, the differences are based on the different metabolic capabilities of fish compared to rats, but also on lower exposure concentrations. In our fish experiments, UV filter levels in water were lower than in rats, exposed to UV filters via feed.

Comparison of in vitro and in vivo activity. The estrogenic activity *in vitro* was matched *in vivo* for most UV filters. 4MBC and OMC exhibited neither estrogenic activity *in vitro* transactivation assays carrying either the hERα or rtERα, nor in fish *in vivo*. 3BC, BP1, BP2 showing activity *in vitro* were also active *in vivo*. Both *in vitro* and *in vivo* they possessed the highest potencies of the tested UV filters. The *in vitro* activity of BP1 (EC50 7.9 x 10^{-7} M) was higher than that of 3BC (1.2 x 10^{-5} M), whereas BP2 was the least active of these three compounds, both *in vitro* and *in vivo* (Figures 1-3). The *in vivo* activity of 3BC was higher than expected from its *in vitro* potency in the rtERα assay, where it was the second potent UV filter after BP1. Being only 87-times less active than E2 in the rtERα assay, BP1 showed only weak *in vivo* activity in fathead minnows. This might be explained by the higher metabolism and lower lipophilicity (and lower bioaccumulation potential) compared to the more lipophilic 3BC. Furthermore, the relatively higher estrogenicity of 3BC *in vivo* might be based on the higher binding activity of 3BC to the ERβ than to ERα of fathead minnows, as 3BC binds preferentially to human recombinant ERβ, and only barely to ERα (Schlumpf *et al.* 2004). There are no indications that metabolites of 3BC are more active than the parent compound. On the basis of the rtERα assay the relative low *in vivo* activity of BP2 is in consistence with our in *vitro data*. This might also be the case for 4DHB, BP3 and BP4 possessing lower rtERα potencies. The estrogenic activity of most benzophenones and salicylates seems to be abolished *in vivo* because of metabolism.

When comparing the potency rankings of UV filters for the rtERα and the hERα assay, the data clearly demonstrates that the rtERα *in vitro* data are more accurate than the hERα data for prediction of the *in vivo* activity. This indicates that hormonal activity of UV filters should

be assessed by a suite of species related *in vitro* and *in vivo* assays, in which the *in vitro* assay should be able to predict to most potent compounds for further *in vivo* testing. Differences *in vitro* and *in vivo* activities, which we nevertheless observed in our fish-based assays, are attributed to metabolism, but also to different activities to different ERs in fish. Our approach using rtERα *in vitro* and fathead minnow *in vivo* may cover species differences in fish. Possibly, using the same fish species (rainbow trout) in the *in vivo* assay as in the *in vitro* assay may have resulted in more comparable results between *the in vitro* and *in vivo* assays.

Environmental consequences. In the environment only a few UV-filters such as OC, 4MBC, BP3 and BM-DBM have been analysed so far. In lake water, BP3, 4MBC and OC occurred at concentrations of 80-125, 60-80 and 22-27 ng/L, respectively, in the upper layer of a bathing lake (Poiger *et al.* 2004), but were lower in other lakes (Balmer *et al.* 2005). Concentrations in treated wastewater were 0.06-2.7 (4MBC), 0.01-0.7 (BP3), 0.01-0.1 (OMC) and 0.01-0.27 µg/L (OC) (Balmer *et al.* 2005). Residues of 4MBC, OMC, BP3, and HMS were also found in muscle tissue of fish from a German lake being between 21-3100 ng/g lipid (sum of all UV filters 2 µg/g in perch and 0.5 µg/g in roach), and between 25-166 ng/g lipid in 10 white fish from Swiss lakes (Balmer *et al.* 2005). However, 3BC, BP1 and BP2 which were found in our study to be estrogenic, have not yet been analysed in aquatic systems. If they were in the same range, VTG induction after short-term exposure to a single UV filter may probably not pose a hazard to fish. However, different UV filters may act additively (Heneweer *et al.* 2005) as indicated for other endocrine disrupters (Routledge *et al.* 1998). Moreover, long-term exposure to UV filters may affect fish reproduction at much lower concentrations.

As it is not known to what extend these UV filters occur in the environment and in fish, comprehensive hazard and risk assessment is premature. Forthcoming studies should determine environmental concentrations of estrogenic UV filters and to relate them to effect concentrations. For hazard and risk assessment, potential effects on reproduction, fecundity and fertility in fish are necessary, as well as bioaccumulation studies. Moreover, UV filters may have multiple hormonal activities such as antiestrogenicity, androgenicity and antiandrogenicity besides estrogenicity (Kunz and Fent submitted). Whether these multiple hormonal activities are reflected *in vivo* in fish and whether reproduction effects occur is currently investigated in our laboratory.

Conclusions

Considering the vast number of compounds to be tested for possible endocrine activity, it is important to employ appropriate *in vitro* systems. They are cost effective and allow for rapid screening of a large number of compounds, but have limitations, which may result in unreliable

predictions. In our present study we show that it is most appropriate to determine the endocrine-disrupting activity of chemicals both *in vitro* and *in vivo*, as no single assay may be best suited to determine the hormonal activity of a compound and because of species differences. We propose that receptor-based assays with related or even the same species should be used for *in vitro* screening prior to *in vivo* testing. In this tiered approach, the predictive power of *in vitro* systems is enhanced and cost intensive *in vivo* studies can be reduced by employing species-specific *in vitro* assays. This leads to the conclusion that an environmental risk assessment should be based on combined, complementing and appropriate species-related *in vitro* and *in vivo* assays for hormonal activity.

Acknowledgment

We thank Farzad Pakdel, Université de Rennes I, Rennes, France, for providing the recombinant rtERα yeast cells, John Sumpter, Brunel University, Uxbridge, U.K., for providing the recombinant hERα yeast cells, Friedrich Jüttner, University of Zürich, and Hans-Rudolf Schmutz, Chemistry Department (FHBB), for support. This work was supported by the Swiss National Science Foundation (NRP50, contract 4050-066554 to K. Fent).

References

Ackermann, G. E., Schwaiger, J., Negele, R. D., and Fent, K. (2002). Effects of long-term nonylphenol exposure on gonadal development and biomarkers of estrogenicity in juvenile rainbow trout *(Oncorhynchus mykiss)*. *Aquat. Toxicol.* **60**, 203-221.

Balmer, M., Buser, H. R., Müller, M. D., and Poiger, T. (2005). Occurrence of some organic UV filters in wastewater, in surface waters, and in fish from Swiss lakes. *Environ. Sci. Technol.* **39**, 953-962.

Blair, R. M., Fang, H., Branham, W. S., Hass, B. S., Dial, S. L., Moland, C. L., Tong, W., Shi, L., Perkins, R., and Sheehan, D. M. (2000). The estrogen receptor relative binding affinities of 188 natural and xenochemicals: Structural diversity of ligands. *Toxicol. Sci.*, 138-153.

Durrer, S., Maerkel, K., Schlumpf, M., and Lichtensteiger, W. (2005). Estrogen target gene regulation and coactivator expression in the rat uterus after developmental exposure to the ultaviolet filter 4-methylbenzylidene camphor. *Endocrinology* **146**, 2130-2139.

Hany, J., and Nagel, R. (1995). Nachweis von UV-Filtersubstanzen in Muttermilch. *Deut. Lebensm.-Rundsch.* **91**, 341-345.

Heneweer, M., Musse, M., Van den Berg, J., and Sanderson, T. (2005). Additive estrogenic effects of mixtures of frequently used UV filters on pS2-gene transcription in MCF-7 cells. *Toxicol. Appl. Pharm.* **In press.**

Holbech, H., Norum, U., Korsgaard, B., and Bjerregaard, P. (2002). The chemical UV-filter 3-benzylidene camphor causes an oestrogenic effect in an *in vivo* fish assay. *Pharmacol. Toxicol.* **91**, 204-208.

Inui, M., Adachi, T., Takenaka, S., Inui, H., Nakazawa, M., Ueda, M., Watanabe, H., Mori, C., Iguchi, T., and Miyatake, K. (2003). Effect of UV-screens and preservatives on vitellogenin and choriogenin production in male medaka (*Oryzias latipes*). *Toxicology* **194**, 43-50.

Jobling, S., Nolan, M., Tyler, C. R., Brighty, G., and Sumpter, J. P. (1998). Widespread sexual disruption in wild fish. *Environ. Sci. Technol.* **32**, 2498-2506.

Kunz, P. Y., and Fent, K. (submitted). Multiple hormonal activities of UV filters in vitro.

Le Guével, R., and Pakdel, F. (2001). Streamlined b-galactosidase assay for analysis of recombinant yeast response

to estrogens. *BioTechniques* **30**, 1000-1004.

Menuet, A., Pellegrini, E., Anglade, I., Blaise, O., Laudet, V., Kah, O., and Pakdel, F. (2002). Molecular characterisation of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. *Biol. Reprod.* **66**, 1881-1892.

Miller, D., Wheals, B. B., Beresford, N., and Sumpter, J. P. (2001). Estrogenic activity of phenolic additives determined by an in vitro yeast bioassay. *Environ. Health Persp.* **109**, 133-138.

Mueller, S. O., Kling, M., Firzani, P. A., Mecky, A., Duranti, E., Shields-Botella, J., Delansorne, R., Borschard, T., and Kramer, P. J. (2003). Activation of estrogen receptor a and ERb by 4-methylbenzylidene-camphor in human an rat cells: comparison with phyto- and xenoestrogens. *Toxicol. Lett.* **142**, 89-101.

Nagtegaal, M., Ternes, T. A., Baumann, W., and Nagel, R. (1997). UV-Filtersubstanzen in Wasser und Fischen. *UWSF-Z. Umweltchem. Ökotoxikol.* **9**, 79-86.

OECD (2002). *Detailed review paper on appraisal of test methods for sex hormone disrupting chemicals*. OECD Environment Directorate, Paris.

OECD (2004). *Detailed review paper on fish screening assays for the detection of endocrine active substances*. OECD Environment Directorate, Paris.

Pakdel, F., Le Gac, F., Le Goff, P., and Valotaire, Y. (1990). Full-length sequence and in vitro expression of rainbow trout estrogen receptor cDNA. *Mol. Cell. Endocrinol.* **71**, 195-204.

Pakdel, F., Métivier, R., Flouriot, G., and Valotaire, Y. (2000). Two estrogen receptor (ER) isoforms with different estrogen dependencies are generated from the trout ER gene. *Endocrinology* **141**, 571-580.

Panter, G. H., Hutchinson, T. H., Länge, R., Lye, C. M., Sumpter, J. P., Zerulla, M., and Tyler, C. R. (2002). Utility of a juvenile fathead minnow screening assay for detecting (anti-)estrogenic substances. *Environ. Toxicol. Chem.* **21**, 319-326.

Petit, F., Valotaire, Y., and Pakdel, F. (1995). Differential functional activities of rainbow trout and human estrogen receptor expressed in the yeast *Sacharomyces cerevisiae*. *Eur. J. Biochem.* **233**, 584-592.

Petit, F. G., Valotaire, Y., and Pakdel, F. (2000). The analysis of chimeric human/rainbow trout estrogen receptors reveals amino acid residues outside of P- and D-boxes important for the transactivation funciton. *Nucleic Acids Res.* **28**, 2634-2642.

Poiger, T., Buser, H. R., Balmer, M., Bergqvist, P. A., and Müller, M. D. (2004). Occurrence of UV filter compounds from sunscreens in surface waters: regional mass balance in two Swiss lakes. *Chemosphere* **55**, 951-963.

Routledge, E. J., Sheahan, D., Desbrow, C., Brighty, G. C., Waldock, M., and Sumpter, J. P. (1998). Identification of estrogenic chemicals in STW effluent. 2. *In vivo* responses in trout and roach. *Environ. Sci. Technol.* **32**, 1559-1565.

Routledge, E. J., and Sumpter, J. P. (1996). Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ. Toxicol. Chem.* **15**, 241-248.

Routledge, E. J., and Sumpter, J. P. (1997). Structural features of alkylphenolic chemicals associated with estrogenic activity. *J. Biol. Chem.* **272**, 3280-3288.

Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B., and Lichtensteiger, W. (2001). In vitro and in vivo estrogenicity of UV screens. *Environ. Health Persp.* **109**, 239-244.

Schlumpf, M., Schmid, P., Durrer, S., Conscience, M., Maerkel, K., Henseler, M., Gruetter, M., Herzog, I., Reolon, S., Ceccatelli, R., Faass, O., Stutz, E., Jarry, H., Wuttke, W., and Lichtensteiger, W. (2004). Endocrine acitivity and developmental toxicity of cosmetic UV filters - an update. *Toxicology* **205**, 113-122.

Schreurs, R. H., Lanser, P., Seinen, W., and Van der Burg, B. (2002). Estrogenic activity of UV filters determined by an in vitro reporter gene assay and in vivo transgenic zebrafish assay. *Arch. Toxicol.* **76**, 257-261.

Schreurs, R. H. M. M., Sonneveld, W., Jansen, J. H. J., Seinen, W., and Van der Burg, B. (2005). Interaction of polycyclic musks and UV filters with the estrogen receptor (ER), androgen receptor (AR), and progesterone receptor (PR) in reporter gene bioassays. *Toxicol. Sci.* **83**, 264-272.

Schultis, T., and Metzger, J. W. (2004). Determination of estrogenic activity by LYES-assay (yeast estrogen screen-assay assisted by enzymatic digestion with lyticase). *Chemosphere* **57**, 1739-45.

Schultz, T. W., Seward, J. R., and Sinks, G. D. (2000). Estrogenicity of benzophenones evaluated with a recombinant yeast assay: Comparison of experimental and rules-based predicted activity. *Environ. Toxicol. Chem.* **19**, 301-304.

Seidlová-Wuttke, D., Jarry, H., and Wuttke, W. (2004). Pure estrogenic effect of benzophenone-2 (BP2) but not of bisphenol A (BPA) and dibutylphtalate (DBP) in uterus, vagina and bone. *Toxicology* **205**, 103-112.

Sohoni, P., and Sumpter, J. P. (1998). Several environmental oestrogens are also anti-androgens. *J. Endocrinol.* **158**, 327-339.

Soto, A. M., Justicia, H., Wray, J. W., and Sonnenschein, C. (1991). p-Nonyl-phenol: an estrogenic xenobiotic released from "modified" polystyrene. *Environ. Health Persp.* **92**, 167-73.

Suzuki, T., Kitamura, S., Khota, R., Sugihara, K., Fujimoto, N., and Ohta, S. (2005). Estrogenic and antiandrogenic

activities of 17 benzophenone derivatives used as UV stabilizers and sunscreens. *Toxicol. Appl. Pharmacol.* **203**, 9 – 17.

Tyler, C. R., Van Aerle, R., Hutchinson, T. H., Maddix, S., and Trip, H. (1999). An in vivo testing system for endocrine disrupting in fish early life stages using induction of vitellogenin. *Environ. Toxicol. Chem.* **18**, 337-347.

Vos, J. G., Dybing, E., Greim, H. A., Ladefoged, O., Lambré, C., J.V., T., Brandt, I., and Vethaak, A. D. (2000). Health effects on endocrine-disrupting chemicals on wildlife, with special reference to the European situation. *Crit. Rev. Toxicol.* **30**, 71-133.

Yamasaki, K., Takeyoshi, M., Yakabe, Y., Sawaki, M., and Takatsuki, M. (2003). Camparison of the reporter gene assay for ER-alpha antagonists with the immature rat uterotrophic assay of 10 chemicals. *Toxicol. Lett.* **142**, 119-131.

Chapter 6

The UV filter 3-benzylidene camphor adversely affects reproduction in fathead minnow (*Pimephales promelas*)

Petra Y. Kunz, Thomas Gries and Karl Fent

accepted in *Toxicological Sciences*

Abstract

The UV filter 3-benzylidene camphor (3BC) is largely used in personal care products and in a number of materials for UV protection. 3BC has been shown *in vitro* and *in vivo* in fish to be estrogenic, but possible effects on fertility and reproduction are unknown. In this study we evaluate whether 3BC affects reproduction of fish *Pimephales promelas*. After a pre-exposure period of 21 days, reproductively mature fathead minnows were exposed to increasing concentrations of 3BC for 21 days in a static-renewal procedure. Actual 3BC concentrations measured by HPLC decreased to about 20% of initial levels and median concentrations were 0.5, 3, 33, 74 and 285 μg/L. 3BC affected reproduction in a dose-dependent manner with an indication of weak effects on fecundity at 3 μg/L, a significant decrease at 74 μg/L and a cessation of reproduction at 285 μg/L. 3BC was accumulated in fish with an average bioconcentration factor of 313±151. Dose-dependent demasculinisation in secondary sex characteristics of male fish and dose-dependent induction of plasma vitellogenin occurred, which was significant at 74 μg/L. 3BC had a profound and dose-dependent effect on the histology of gonads of males and female fish. At 74 and 285 μg/L oocyte and spermatocyte development was inhibited in male and female gonads. Testes of exposed males had much fewer spermatogenic cysts, and ovaries of exposed females had much fewer mature, and more artretic follicles. This study shows significant effects of the common UV filter 3BC on fertility, gonadal development and reproduction of fish after short-term exposure that may have negative consequences on the population level.

Introduction

UV filters are increasingly used in cosmetics, skin and hair care products like sunscreens, shampoos, creams and fragrances. They are also applied in textiles, optical material and optics components, household products, fabrics, transdermal drug delivery systems and for UV stabilization of a variety of materials. By absorbing, scattering and reflecting UV light they protect the human skin and materials from the negative effects of UV radiation. The amount of UV filters added are increasing, because higher sunlight protection factors are used, and thus generally higher percentages of different UV filters are required in the products.

Consequently it is not surprising that residues of UV filters have been detected in the environment. They enter the aquatic system either directly into surface water via recreational activities (bathing) or indirectly via wastewater. In lakes and wastewater they were detected at concentrations up to 125 ng/L (Poiger et al., 2004) and 19 μg/L (Balmer et al., 2005), respectively. Because they are photostable, often lipophilic (log Kow 3-7), UV filters are relatively stable in the aquatic environment (Poiger et al., 2004; Balmer et al., 2005) and thus critical for bioaccumulation. Indeed, residues of several UV filters have been detected at concentrations of 21-3100 ng/g lipid in fish (Nagtegaal et al., 1997; Balmer et al., 2005; Buser et al., 2006) and even in human milk (Hany and Nagel, 1995).

Besides being present in the environment, UV filters recently gained increasing environmental relevance because of their reported estrogenic activity *in vitro* (Routledge and Sumpter, 1997; Schultz et al., 2000; Schlumpf et al., 2001; Schreurs et al., 2002; Mueller et al., 2003; Kunz et al., 2006; Kunz and Fent, in press; Kunz and Fent, submitted) and *in vivo* in rats (Schlumpf et al., 2004a; Schlumpf et al., 2004b). In particular the fact that estrogenic activity has been observed in fish *in vitro* (Kunz and Fent, in press) and *in vivo* (Kunz et al., 2006) makes UV filters of potential environmental concern for aquatic organisms. 3-Benzylidene camphor (3BC), benzophenone-1 and benzophenone-2 led to vitellogenin induction in juvenile fathead minnows (Kunz et al., 2006), and this was also found for 3BC in rainbow trout (Holbech et al., 2002), 4-methyl benzylidene camphor (4MBC) and octyl-methoxycinnamate (OMC) in male medaka (Inui et al., 2003). Among the UV filters 3BC showed highest estrogenic activity *in vivo* in fish (Kunz et al., 2006), but also in rats (Schlumpf et al., 2004a; Schlumpf et al., 2004b).

Currently, it is not known whether UV filters affect fecundity of fish. This question is of ecotoxicological and ecological importance because UV filters are present in the aquatic environment and may possess enough potency to adversely affect reproduction and development in fish. Estrogenic effects in several fish species was shown for many chemicals, and for sewage effluents (Harries et al., 1997; Tyler et al., 1998; Rodgers-Gray et al., 2001; Tilton et

al., 2002; Sumpter and Johnson, 2005). In the United Kindom widespread sexual disruption was observed in wild roach (Jobling et al., 2002), likely due to chemicals in sewage. Several different groups of chemicals were found to be estrogenic in short-term exposures by inducing VTG in juvenile and male fish. Subsequently they were demonstrated to negatively affect reproduction, sometimes at environmentally relevant concentrations such as ethinylestradiol (Nash et al., 2004), 4-tert-octylphenol (Gronen et al., 1999), and bisphenol A (Haubruge et al., 2000), to name a few.

In this work, we evaluate whether a common UV filter, 3BC, adversely affects reproduction in fathead minnows. This UV filters has practical importance as it is added to skin and hair care products (allowed up to 2% per weight in Switzerland and EU), but also to household products, fabrics, and other products. We investigated important parameters for endocrine disruption, such as fecundity, vitellogenin induction, secondary sex characteristics and gonad histology. The findings of this study show for the first time that a commonly used UV filter adversely affects reproduction in fish in a dose-dependent manner after short-term exposure.

Material and Methods

Chemicals. 3-Benzylidene-camphor (3BC, MG 240.34, CAS15087-24-8) was from Induchem (Volketswil, Switzerland) and 17β-Estradiol (E2) was purchased from Fluka AG (Buchs, Switzerland). All compounds were >99% pure. Stock solutions were made in N,N-dimethylformamide (DMF) and stored in the dark at 4°C. Analytical grade DMF was purchased from Arcos Organics (Geel, Belgium).

Fish. Fathead minnows are found as wildfish throughout much of North America and have been chosen because of short reproductive cycles, frequent use in endocrine disrupter research and established techniques including vitellogenin (VTG) antibodies (Parks et al., 1999). We used reproductively, mature fathead minnows (*Pimephales promelas*) between 8-9 months of age. Fish have not been held in a culture situation conductive to routine spawning before the onset of the experiment.
Newly mature fathead minnows were received from the cultivator (Aquatic Research Organisms, Hampton NH, USA) and adapted for a minimum of 14 d in our laboratory in a tank prior to the experiment. Fish were fed with Tetramin pellets (Tetra GmbH, Melle, Germany) twice a day with a quantity of 1% of body weight prior to the onset of experiments. During the experiments, fish were fed with brimeshrimp (*Artemia salina*, Argent Chemical Labs, Redmond WA, USA) at a feeding rate of 1% of body weight twice a day.

Exposure. The experimental procedure was similar to that described recently (Harries et al., 2000; Ankley et al., 2001; Panter et al., 2002). During the pre-exposure and exposure period fish were held 10 L stainless steel aquaria in well-aerated reconstituted tab water medium (total hardness 160 mg/L as $CaCO_3$, total alkalinity 30 mg/L as $CaCO_{3,}$ conductivity 500 µs/cm) and a 16 h/8 h light/dark cycle at 25±1°C. A 48 h static-renewal procedure was used, renewing the total of aquaria water (10 L). After 24 hours, food remnants and faeces were removed by siphoning a third of the water volume and replacing it by new water containing the appropriate concentrations of 3BC. This administration regime was chosen because of the high lipophility of 3BC (Log-Kow = 5.37), which made it not possible to deliver it by a flow-through system. In order to ensure normal reproductive performance of the fish, unnecessary disturbances were minimized by a static-renewal regime of 48 h instead of 24 h. Thus water quality was monitored continuously in order to enable conformance with performance-based criteria (OECD, 2001). Aliquots of 250 mL water were taken for chemical analysis.

At the beginning of the experiment, four females and two males were randomly assigned to the replicate stainless steel tanks (10 L). Prior to the exposure period, a pre-exposure phase was initiated in order to establish the reproductive capacity of females and to provide a tank-specific baseline data for potential statistical comparison after initiation of chemical exposure. During pre-exposure and exposure period survival, appearance and behavior of fish, reproductive behavior, secondary sex characteristics and fecundity (cumulative number of spawned eggs) were determined. Once successful spawning has been established, generally after 14 to 21 days, 3BC exposure was started. Fish were exposed for 21 days to nominal concentrations of 1, 10, 100, 250 and 500 µg/L 3BC. A control and a solvent control (SC, 1 mL DMF in 10 L water) were also included. Stock solutions of 3BC was prepared freshly in DMF prior to the start of the experiment and added to the experimental water by mixing. The 3BC concentrations were selected according to concentrations which were found to induce vitellogenin in juvenile fathead minnows (Kunz et al., 2006). For the selected concentrations of 3BC no toxic side effects (i.e. lethargy, uncoordinated swimming, loss of equilibrium, hyperventilation) were observed.

Physico-chemical measurements and biological observations. Physico-chemical parameters were determined daily. pH and oxygen saturation ranged between 7.2–7.9 and 6.5–8.3 mg/L, respectively, throughout the exposure period. Mortalities and abnormal behaviour were recorded daily and dead fish were removed from the tanks. At the end of the experiment (day 21 of the exposure period) all fish were anaesthetized with buffered tricaine methan sulfonate (MS-222, 100 mg/L with 200 mg $NaHCO_3$/L). Subsequently the length of individual fish was measured, they were weighted and measures of a number of endpoints were made. In order to determine plasma concentrations of vitellogenin, blood was collected from the caudal vein using a heparinised capillary tube (KABE Labortechnik GmbH, Nümbrecht-Elsenroth, Germany)

and transferred into a labelled Eppendorf® tube. Plasma was then separated from the blood by centrifugation (10 min. at 3'000 rpm at 4°C) and stored until analysis with protease inhibitors (Aprotinin, Fluka AG, Buchs, Switzerland) at -80 °C. After sampling of the blood gonads were removed and weighted for determination of the gonad-somatic index (GSI = 100 x gonad weight / body weight). Gonads were then placed in Buoin's solution (Fluka AG, Buchs, Switzerland) for subsequent histological analysis. Photographs of the head (number of nuptial tubercles) of male fathead minnows were taken for further analysis of the secondary sexual characteristics. The remainder of the carcass of the fish was frozen at -80°C for chemical residue analysis.

Vitellogenin analysis. Plasma samples were thawed on ice and ice-cold assay buffer (Biosense, Bergen, Norway) was added to the individual samples in order to achieve a 1:2 ratio of plasma: buffer. The samples were vortexed and immediately used for vitellogenin (VTG) analysis. For quantification of VTG a commercially available, homologous enzyme-linked immunosorbent assay (ELISA) for fathead minnow VTG was used as described by Biosense Laboratories AG (Bergen, Norway). Purified fathead minnow VTG from blood plasma (Biosense AG) was used as a standard for quantitation according to the provider's description.

Histology. Routine histological procedures were used to analyse testes and ovaries of the fish. The gonads from the Buoin's solution were embedded in paraffin. Sections were taken along the long axis of the gonad at 6 µm intervals, in a serial step fashion. Two serial sections were collected from 3 steps equally spaced between the leading edge of the tissue and the midline of the gonad, for a total of 6 tissue sections/sample. The sections were stained with Haemalaun (Mayer's Haemalaun; Merck, Darmstadt, Germany) and Eosin (Fluka AG, Buchs, Switzerland). Evaluation of the histological effect of 3BC on gonads was based on staging of ovarian and testicular development (similar to e (Leino et al., 2005). The ovary was evaluated based upon relative frequencies of oogonia, early vitellognic, late vitellogenic and artretic follicles. Frequencies were evaluated by counting the different stages in three randomly selected fields of vision/female (90x magnification). Testis staging was based on the relative frequency of spermatogonia, spermatocytes and spermatides present. Thereby for each male pictures of four randomly selected fields of vision (19'276 μm^2) were taken and the areas occupied by the different stages were measured.

Analytical chemistry. During the experiment 250 mL of exposure water were taken three times from all treatment groups at the beginning (0 h), after siphoning and renewal of the siphoned water (24 h) and prior to water renewal (48 h). The water samples were stored in brown glass flasks at 4°C until analysis by HPLC-DAD. Briefly, 25 or 250 mL of water samples, depending on sample concentration, were extracted and concentrated by solid phase extraction (SPE), using Sep-Pak Vac 3cc C18 cartridges (Waters AG, Rupperswil, Switzerland). Cartridges were conditioned wit dichloromethane, methanol and H2O bi-destilled prior to the extraction

of the water samples. The cartridge was then air-dried and eluted with dichlorotmethane. Dichloromethane was allowed to evaporate to dryness and the dry eluent was resuspended in 100 µL of ethanol and analysed by HPLC-DAD (Agilent 1100 Series). Thereby a concentration of 250 to 2'500-times of the water sample was achieved.
The remainders of the fish carcasses were analysed for 3BC residues after the 21-day exposure period by means of a liquid-liquid extraction. Briefly, single fish were homogenized together with bidestilled water and ethylacetat in a ratio of 1:2:2 (fish:water:solvent). The homogenate was vortexed until it was mixed thoroughly and then centrifuged (15 min, 4°C, 4'500 rpm). The supernatant (3BC solved in ethylacetat) was transferred to another vial and then the solvent was evaporated to dryness. The dry residue was resuspended in 1 mL of ethanol followed by another centrifugation step (30 min, 16'000 rpm). The supernatant was then transferred to a brown GC vial and analysed by HPLC-MS.

Data processing and statistical analysis. The data distribution was tested for normality by using the Kolmogorov-Smirnov test. Differences between treatments were assessed using either analysis of variance (ANOVA) followed by a Dunnett's Multiple Comparison test to compare the treatment means with respective controls, or a non-parametric test, when a normality distribution of the data was not given. In this case differences between treatments were assessed by a Kruskal-Wallis test followed by a Dunn's Multiple Comparison test to compare the treatment means with respective controls. Statistical comparisons were made with the sovent control as the overall control, given that the results for comparisons with the water control lead to the identical findings. The results are given as mean ± standard error of mean (SEM). Differences were considered significant at $p \leq 0.05$. All computations were performed with PRISM 4.0 (GraphPad Software Inc., San Diego, USA).

Results

Actual 3BC concentrations in aquaria waters and concentration in exposed fish. Water concentrations of 3BC were determined by HPLC-DAD measured at three time points (0, 24, and 48 h) at all exposure concentrations in order to determine actual effect concentrations. Table 1 shows that actual concentrations at the beginning of the exposure (0 h) were close to nominal values (mean = 112.0±6.9%, n=5). During exposure 3BC concentrations decreased to a similar extent for all concentrations. After 24 h at partial water renewal 3BC concentrations decreased to 30-57 %, and after 48 h before water renewal, to 20-32% of nominal concentrations. The decrease is due to several factors including absorption on aquaria walls, spawning substrates, food, fish and eggs, as well as bioaccumulation of 3BC by fish and spawned eggs. To determine the bioaccumulation potential of 3BC whole body residues of 3BC in fish were measured at

Table 1. Nominal and measured concentrations of 3BC in exposure waters and fish after 21 days of exposure.

EXPOSURE WATERS								FISH	
Nominal	Measured							Whole Body	BCF*
	0 h		24 h (µg/L)		48 h (µg/L)		Median	Concentration	
(µg/L)	(µg/L)	(%)	(µg/L)	(%)	(µg/L)	(%)	(µg/L)	(µg/g)	
1	1.1 ± 0.1	101%	0.5 ± 0.1	45%	0.3 ± 0.1	27%	0.5	0.16 ± 0.18	399
10	9.9 ± 0.3	99%	3.1 ± 1.1	31%	2.0 ± 0.4	20%	3	0.36 ± 0.27	102
100	118 ± 5	118%	44 ± 3	37%	22 ± 0.2	18%	33	7.22 ± 3.17	230
250	293 ± 34	117%	74 ± 3	25%	56 ± 1	19%	74	36.49 ± 21.91	493
500	547 ± 19	109%	285 ± 14	52%	160 ± 7	29%	285	100.73 ± 47.87	344

the end of the experiment after 21 days. Table 1 shows that whole body residues of 3BC in adult fathead minnows were between 0.16 and 100.7 µg/g depending on the exposure concentration. Based on mean actual water concentrations, this gives an average bioconcentration factor of 313±151.

Fish survival, weight and length.

We observed identical survival rates (94.4±9.6 %, n=3 replicates) for the control fish and all exposed fish, except for the fish exposed to 33 µg/L 3BC (100 %, n=3 replicates) and 285 µg/L 3BC (88.9±9.6 %, n=3 replicates). However, survival did not differ significantly from the control tanks (p = 0.9306). No differences were observed in length of males and females or in weights of males and females at the end of the experiment (Table 2).

Table 2. Body weight and length of exposed fish after 21 days of exposure.

	Exposure (µg/L)	Body weight (mg)	Body length (mm)
Controls	Water	211.3 ± 91.1	29.4 ± 2.8
	Solvent	238.0 ± 88.8	30.8 ± 3.5
3BC	0.5	335.9 ± 128.9	32.8 ± 3.1
	3	258.9 ± 95.9	30.9 ± 3.4
	33	115.1 ± 37.4	24.7 ± 2.4
	74	95.8 ± 25.8	23.7 ± 1.8
	285	171.4 ± 35.0	25.9 ± 1.8

Effects on reproduction. The cumulative fecundity, i.e. the cumulative number of eggs/ treatment, differed slightly for all treatments and replicates before exposure (Fig. 1). At the start of 3BC exposure, none of the fish of the water and solvent control replicates stopped spawning (Fig. 2A). At the lowest exposure concentration of 0.5 µg/L 3BC, fish in one of the three aquaria stopped spawning after 8 days of exposure, whereas fish in the other two replicates continued with slightly reduced spawning activity (Fig. 2B). At 3 µg/L 3BC, fish of one aquarium stopped spawning after 8 days, whereas fish of the other aquaria continued with reduced spawning activity compared to pre-exposure and controls (Fig. 2C). At 33 µg/L 3BC,

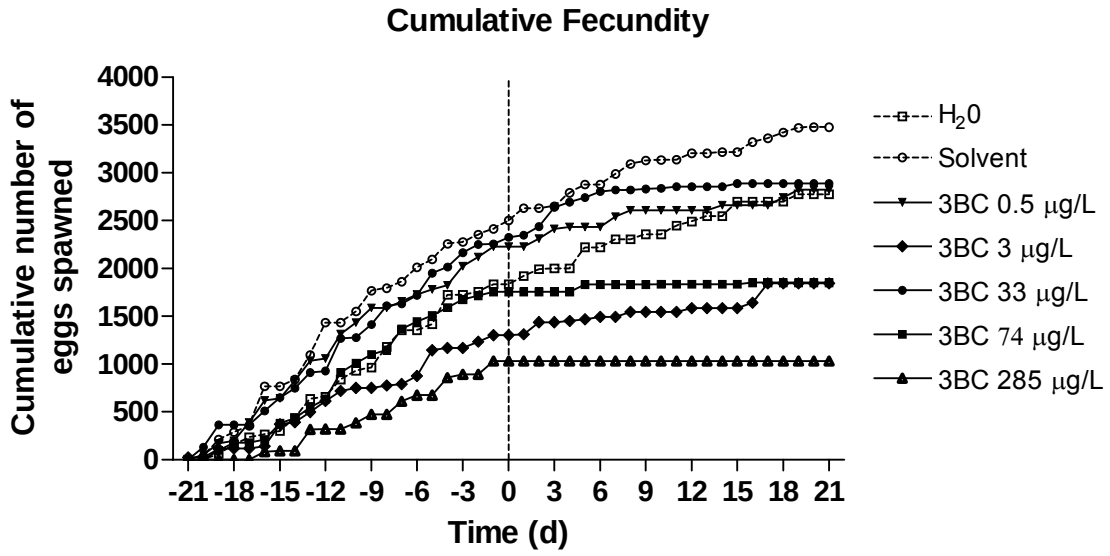


Figure 1 Cumulative number of eggs spawned per treatment group during pre-exposure (days -21 to 0) and exposure period (days 0 to 21). Treatments are given in measured median concentrations.

the fish in two of the three aquaria stopped spawning after 4 and 7 days, respectively, whereas the third aquarium showed reduced spawning with less spawns and eggs/spawn (Fig. 2D). Fish in one aquarium exposed to 74 µg/L 3BC stopped spawning activity immediately, whereas the fish of the second and third aquarium spawned only once after the onset of exposure (Fig, 2E). All fish exposed to 285 µg/L 3BC ceased spawning activity immediately (Fig 2F). Overall, 3BC lead to a dose-dependent inhibitory effect on fertility and reproduction in fathead minnows. This was already observable at 0.5 µg/L 3BC, but was not significant. Figure 3 A-C shows that the number of spawns, number of eggs/spawn and the number of eggs/female/day were significantly decreased at 74 and 285 µg/L (ANOVA $p > 0.05$).

In male fathead minnows exposure to 3BC lead to a dose-dependent VTG induction, which was significant at 74 and 285 µg/L 3BC (Fig. 4). In these fish mean plasma VTG content was between 5'272 and 18'020 µg/mL compared to the water and solvent control having a mean residual level of 15 µg/mL VTG. In female fish no significant VTG induction was observed. Mean levels ranged between 1'300 and 5'400 µg/mL for all treatment groups (Fig. 4). Moreover, exposure to 3BC caused alterations in male secondary sexual characteristics (Fig. 5). The number of tubercles significantly decreased at 74 and 285 µg/L, whereas the remaining tubercles appeared to be less pronounced (Fig. 5). The decrease in the number of tubercles was dose-dependent (Fig. 6). At 33 µg/L 3BC and higher tubercles were less in numbers and seemed less pronounced.

Neither in males, nor in females GSI was altered (data not shown). However, gonadal histology was profoundly affected by 3BC. This UV filter induced a dose-dependent inhibition of spermatogenesis in the testis of male fish. Even at concentrations of 3 µg/L 3BC, testes of

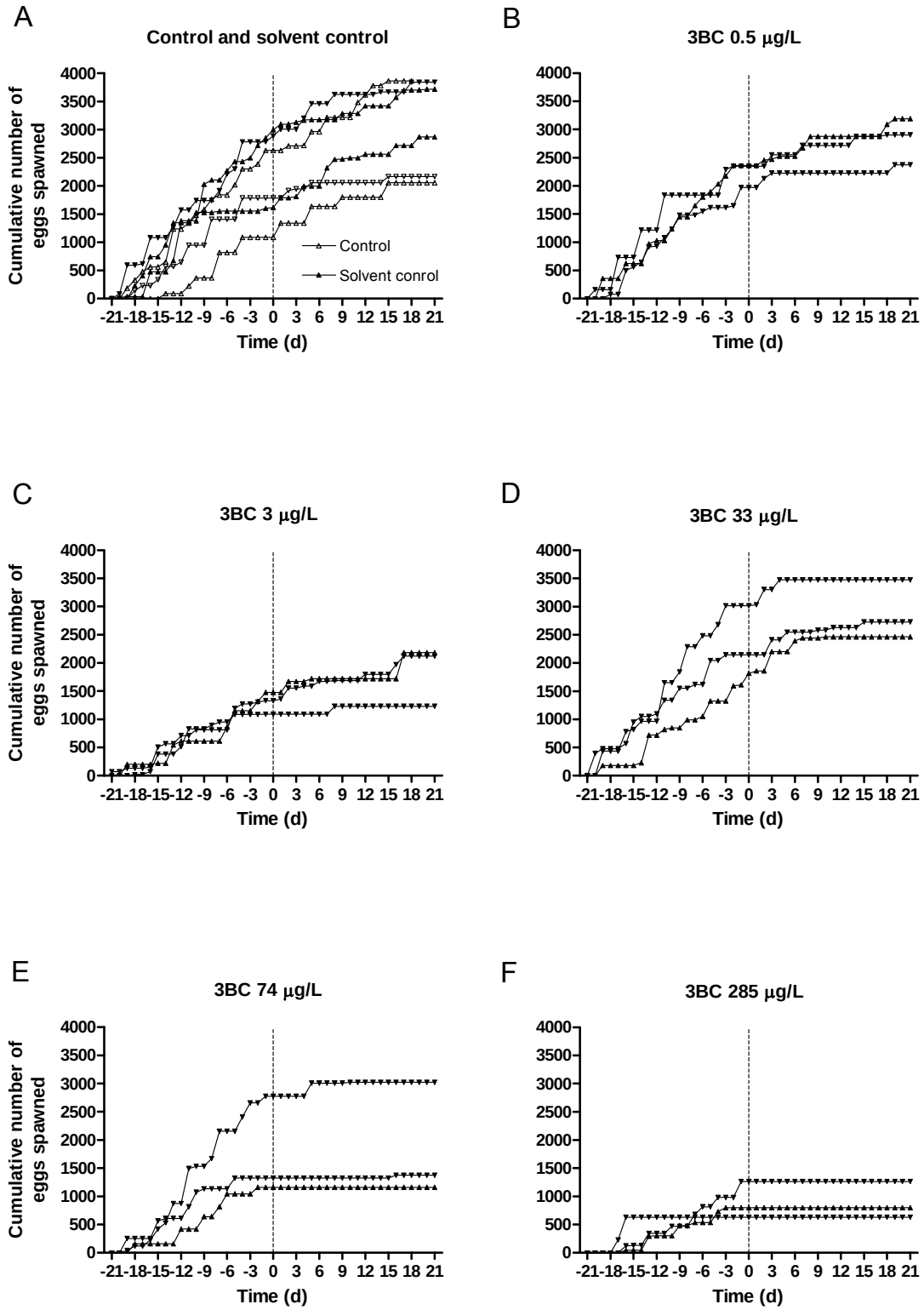
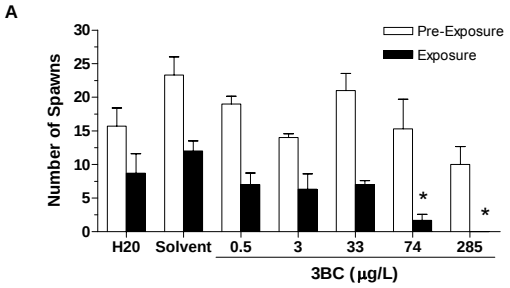
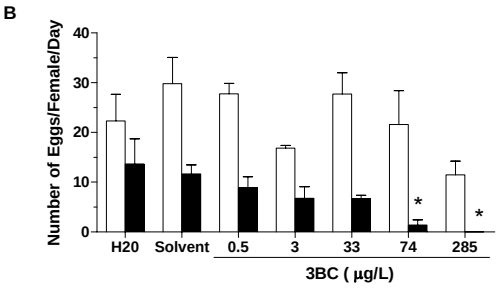


Figure 2 Cumulative number of eggs spawned in each of the three replicates during pre-exposure (days -21 to 0) and exposure period (days 0 to 21), shown for controls and each treatment group (A-G). Measured median 3BC concentrations are given.





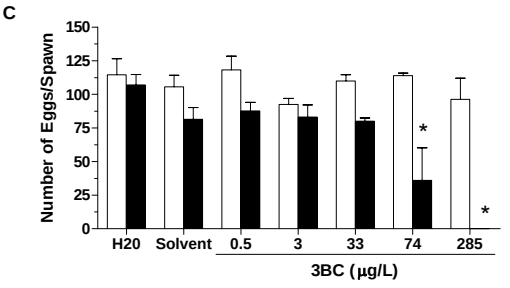


Figure 3 Fecundity shown as percentage of eggs and spawns during exposure (black bars) relative to pre-exposure (100%)(white bars). Number of spawns (A). Number of eggs/female/day (B). Number of eggs/spawn (C). Data shown are means and SEM (3 replicates/treatmet). Asterics denote significant difference from control at $p \leq 0.05$. Measured median 3BC concentrations are given.

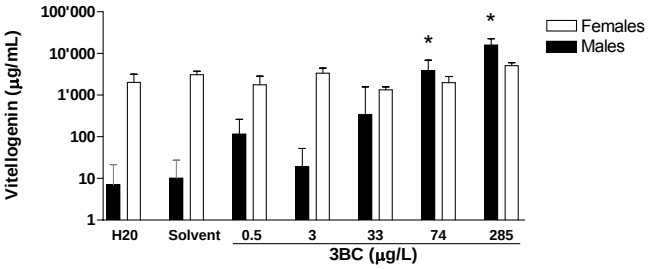


Figure 4 Vitellogenin concentration in female and male fathead minnows exposed to 3BC. Values are means ± SEM (females: n=12, males: n=6). Asterics denote significant difference from control at $p \leq 0.05$. Measured median 3BC concentrations are given.

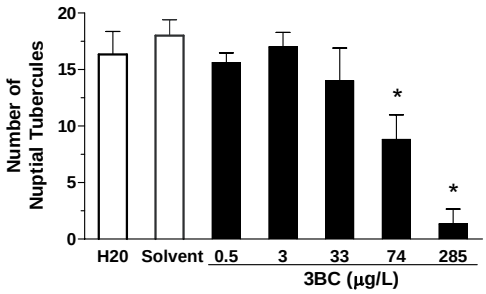


Figure 5 Number of nuptial tubercles in male fathead minnows exposed to 3BC. Values are means ± SEM (n=6). Asterics denote significant difference from control at $p \leq 0.05$. Measured median 3BC concentrations are given.

exposed males clearly differed from control testes, possessing reduced tubules with fewer spermatocytic stages. This effect increased with increasing concentrations, and at 74 and 285 µg/L spermatocytic stages decreased by 30 to 40 % compared to the control. Spermatogenesis appears to be inhibited and testes were characterized by enlarged semiferuous tubules filled with sperms. Moreover, there is a relative lack of germinal epithelium and primary and secondary spermatocytes. Spermatognia apparently did not undergo a further differentiation to spermatogenic cysts at 3 µg/L 3BC and higher concentrations ($p < 0.001$). Spermatocytes were less present, whereas the area occupied by spermatides increased in a dose-dependent manner (Fig. 7A and 8).

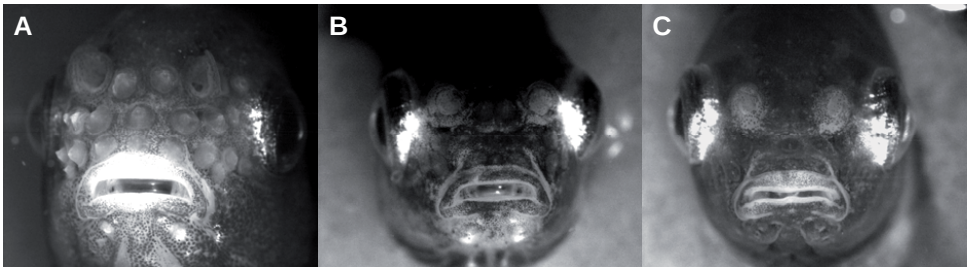


Figure 6 Nuptial tubercles of male fathead minnows. A) Control male. B) Male exposed to 74 μg/L 3BC. C) Male exposed to 285 μg/L 3BC.

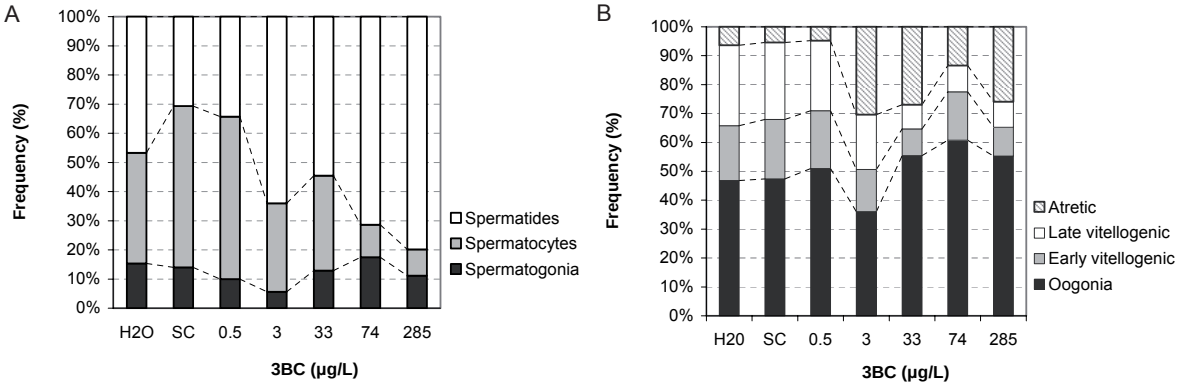


Figure 7 Relative percentage of the different stages of spermatids in testis of male (A) and oocytes in ovaries of female (B) fathead minnows after 21 days of exposure to 3BC. For males, n = 4 to 5, for females, n = 4 to 6.

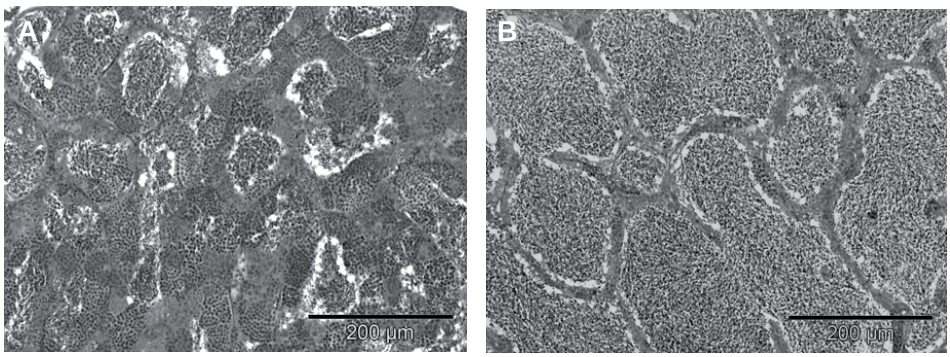


Figure 8 (A) Section of testis from a control male, typical of active spawning. (B) Section of testis from a male exposed to 285 µg/L 3BC. Note the enlarged seminiferous tubules filled with sperms and relative lack of germinal epithelium in the tubules.

In ovaries of treated females exposed to the lowest concentration of 0.5 µg/L 3BC no discernible differences between treated and control females could be seen. Ovaries of exposed females to 3 µg/L 3BC and higher concentrations led to an increase in the number of artretic follicles up to 24%, accompanied by a decrease in early and late vitellogenic stages (Fig. 7B and 9). Late vitellogenic follicles decrease by 8% at 3 and 33 µg/L 3BC, but then remained stable in number at 74 and 285 µg/L. At 33 µg/L and higher, the numbers of oogonia increased (Fig. 7B). These histological alterations in gonads of male and females fish demonstrate a dose-dependent profound estrogenic effect of 3BC.

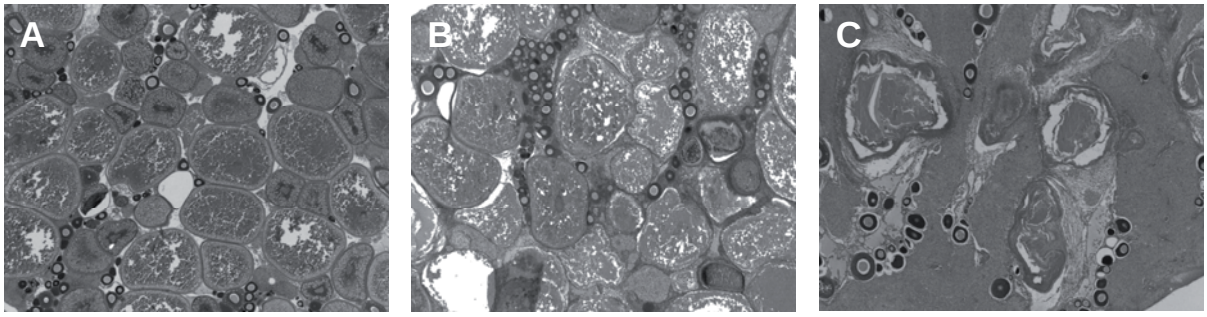


Figure 9 (A) Section of control ovary, showing mature follicles typical of an actively spawning female. (B-C) Section of ovaries from females exposed to 285 µg/L, showing increased number of artretic follicles (B) and degenerated ovary with only early stages of oogonia (C).

Discussion

The UV filter 3BC is used in many products such as sunscreens, skin and hair care products, household products, textiles, fabrics, optical materials and components and transdermal drug delivery systems. Although 3BC enters the aquatic system either directly into surface or indirectly via wastewater, environmental concentrations are not yet known. However, its lipophilic nature makes 3BC prone for bioaccumulation in aquatic organisms. 3BC was shown to have estrogenic activity *in vitro* (Holbech et al., 2002; Kunz et al., 2006) and *in vivo* leading to significant vitellogenin induction in fish (Holbech et al., 2002; Kunz et al., 2006). So far, it was unknown whether 3BC affects reproduction in fish in addition.

In the present study, we show for the first time that a commonly used UV filter adversely affects reproduction in fish in a dose-dependent manner. Immediately after the onset of exposure to 285 µg/L 3BC spawning ceased completely, and it was significantly reduced at 74 µg/L. Fish that were successful spawners during the pre-exposure period had no or significantly less spawns with significantly fewer eggs per spawn when exposed to 3BC. The immediate and strong impact of 3BC on spawning activity at the highest exposure concentration was surprising. This also holds for the clear effect at 74 and 285 µg/L. At the lowest exposure concentration of 0.5 µg/L fish showed slightly reduced spawning activity and one replicate even stopped spawning after eight days of exposure, which indicates adverse effects of 3BC on reproduction, although not being significant.

.

These dose-dependent effects down to the lowest exposure concentration were also observed in gonadal histology. Even at 3 µg/L testes of exposed males were clearly distinguishable from control testes, and possessed reduced tubules with fewer spermatocytic stages. This effect increased with increasing concentrations, and at 74 and 285 µg/L, spermatocytic stages

decreased by 30 to 40% compared to the control. Spermatogenesis appears to be inhibited and testes were characterized by enlarged semiferuous tubules filled with sperm and the relative lack of germinal epithelium and primary and secondary spermatocytes. Spermatognia apparently did not undergo a further differentiation to spermatogenic cysts. The fact that also egg production ceased so quickly suggests a standstill of milt production indicated by the accumulation of sperms in the lumen of enlarged semiferous tubules and the lack intermediate stages (spermatogenic cysts).

The histological response of testes to 3BC is much like those observed in previous studies with E2 and EE2. Whereas these estrogens lead to a full inhibition of testicular development, depending on the dose from a degeneration of spermatozoa to total atrophy (Gimeno et al., 1998; Miles-Richardson et al., 1999a; Palace et al., 2002; Pawlowski et al., 2004). The 21-days exposure to 3BC also inhibited testicular development, but showed less degeneration. Similar inhibition of testicular development was recently reported for fish exposed to the weak estrogen receptor agonist 4-nonylphenol, which lead to a significant reduction of fecundity (Miles-Richardson et al., 1999b; Harries et al., 2000) and a significant necrosis of sperm cells and spermatozoa (Miles-Richardson et al., 1999b). Although we did not investigate the state of sertoli cells, the presence of phagocytic cells in the semiferous tubules and of macrophages in their lumina indicates an extension of the phagocytic role of these cells in males exposed to high concentrations of 3BC (Smith, 1974; Russell et al., 1990; Miles-Richardson et al., 1999a).

In ovaries the histological effects of 3BC were just as pronounced. Whereas at 0.5 µg/L 3BC no discernible differences between treated and control females could be seen, 3 µg/L 3BC and higher concentrations led to an increase in the number of artretic follicles up to 24%, accompanied by a decrease in early and late vitellogenic stages. Interestingly, from 3 to 33 µg/L 3BC late vitellogenic follicles decrease by 8% but then remained stable in number at 74 and 285 µg/L. This may be an indication that the strong inhibition of reproduction at the two highest exposure concentrations might be affected by the testicular status of the males, where spermatogenesis was increasingly inhibited with increasing exposure concentrations of 3BC.

Strong estrogen receptor agonists such as E2 and EE2 have similar effects on ovaries (Kramer et al., 1997; Miles-Richardson et al., 1999a; Länge et al., 2001). Similar to these estrogens, 3BC seems to interfere with egg production and lead to fewer mature, and more artretic follicles in treated ovaries of female fathead minnows. Possibly sustained abnormally high vitellogenin levels induced by estrogen receptor agonists interfere with final maturation and release of oocytes from the ovary, hypothetically by inhibiting gonadotropin II release by the pituitary (Kramer et al., 1997; Miles-Richardson et al., 1999a). High concentrations of weak estrogen

receptor agonists such as methoxychlor also resulted in increased follicular artresia in some females (Ankley et al., 2001), whereas nonylphenol did not induce any histological changes in ovaries (Miles-Richardson et al., 1999b).

The estrogenicity of 3BC was manifested not only in a dose-dependent reduction of spawning activity caused by gonadal degeneration, but also by VTG induction and feminization of male secondary sex characteristics. Significant VTG induction in male fathead minnows was observed at 74 µg/L 3BC. This was observed after 14 days of exposure to 435 µg/L (Kunz et al. 2006), but also for other estrogenic chemicals acting via the estrogen receptor, such as the pesticide methoxychlor (Ankley et al., 2001). VTG induction in 3BC exposed fish goes in line with a significant decrease in fecundity. The cumulative number of eggs spawned and gonadal histology are considerably more sensitive to the effect of 3BC, contrary to methoxychlor, where VTG induction turned out as the most sensitive endpoint. No significant VTG induction occurred in females, similar to methoxychlor (Ankley et al. 2001). However, when female fathead minnows are exposed to strong synthetic estrogens, a sustained increase in plasma vitellogenin occurred (Kramer et al., 1997; Miles-Richardson et al., 1999a). The lack of siginificant VTG increase above the already high VTG level in reproductive females is an indication of the weaker estrogenicity of 3BC compared to EE2.

Besides the reduced fecundity and the clear VTG induction, in males the significant decrease of the number of tubercles in male fish was a clear indication for the estrogenic activity of 3BC. Males at the highest exposure concentration were visually not discernible from females and all but one have lost all tubercles. Nuptial tubercles in the male fathead minnow can decrease when exposed to both, the weak ER agonists 4-nonylphenol (Miles-Richardson et al., 1999a; Miles-Richardson et al., 1999b; Harries et al., 2000) , and the strong ER agonists E2 and EE2 (Miles-Richardson et al., 1999a; Pawlowski et al., 2004). The development of nuptial tubercles and the fatpad in male fathead minnows is stimulated by testosterone produced by the Leydig cells of the testes (Smith, 1978). Testosterone production is under the control of FSH and LH, both of which are regulated by E2 in a feedback mechanism (Junqueira et al., 1986). Thus athropy of the nuptial tubercles may have resulted from an inhibition of LH because xenostrogens like 3BC may suppress androgen levels by altering neuroendocrine feedback loops (Trudeau et al., 1993; Jobling et al., 1996; Ankley et al., 1998). The number of tubercles, as well as the production of sperms are androgen-dependent processes, they are negatively correlated with exposure to estrogenic chemicals (Smith, 1974; Miles-Richardson et al., 1999a; Jensen et al., 2001). Thus, demasculinisation reflected by these endpoints is an indication for the estrogenic activity of 3BC.

Different effects on nuptial tubercles are observed with other compounds. The anti-androgen flutamide, which has a limited binding capacity to fish androgen receptors (Wells and Van Der

Kraak, 1998; Sperry and Thomas, 2000) causes a significant decrease in number of tubercles in male fathead minnows at high concentrations, but does not induce VTG production in males. On the other hand the aromatase inhibitor fadrozole leads to a significant VTG induction in males and females, and to a significant tubercles induction in female fish (Ankley et al., 2002).

Reduced reproduction and even its cessation caused by 3BC may be related to alterations in both males and females. In males we observed a dose-dependent demasculinisation, indicated by the loss of secondary sexual characteristics, and the inhibition of spermatogenesis. This already started at 3 µg/L, and possibly resulted in a loss of gender specific mating behavior and in a stop of milt reproduction at 74 and 285 µg/L. In females, the inhibition of oogenesis started at 3 µg/L 3BC and was indicated histologically by an increase of artretic and a decrease of early and late vitellogenic follicles in ovaries. At 74 and 285 µg/L females stopped egg production and the release of mature oocytes. Possibliy this may be a reaction on the missing mating behaviour of demasculisized males present in the aquaria.

So far UV filters have been evaluated for estrogenic activity in fish by investigating VTG induction only. This was observed in juvenile fathead minnows for 3BC, BP1 and BP2 (Kunz et al., 2006), and also found after injection of 3BC (68 mg/kg) in rainbow trout (Holbech et al., 2002), and after aqueous exposure to 100 mg/L 4MBC and 10 mg/L octyl-methoxycinnamate in male medaka (Inui et al., 2003). The benefit of measuring VTG induction as a biomarker for estrogenicity is obvious in our study. It is easier to measure and provides information of the estrogencity of a certain compound with possible adverse effects on reproduction (Tyler et al., 1999; Sumpter and Johnson, 2005). The lowest observed effect concentrations after exposure to 3BC vary according to the endpoint. For VTG induction and secondary sex characteristics it was 74 µg/L, for effects on fecundity 33 µg/L, and histological alterations occurred already at 3 µg/L. VTG determinations in short-term experiments are rather fast, and less manpower- and time-consuming than a short-term reproduction test, but our results with 3BC suggest, that it posses lower sensitivity than other parameters. Nevertheless VTG induction is clearly associated with other direct markers of reproduction effects and thus well suited for screening estrogenic compounds (Tyler et al., 1999; Sumpter and Johnson, 2005). However, for hazard and risk assessments, short-term reproduction assays provide much more important toxicological and ecological information. They give a more detailed picture of the endocrine activities of a compound and give sensitive information on fecundity, histology and secondary sex characteristics, and thereby facilitate and refine risk assessment.

Conclusions and environmental consequences

Our study demonstrates significant effects of 3BC on the reproduction of fathead minnows. The lowest observed effect concentrations for the most sensitive parameter, gonadal histology, was 3 µg/L. Environmental concentrations of 3BC in water and fish have not yet been reported, but may reach the same level than other UV filters such as 4MBC. If environmental concentrations of 3BC are also in the low ng to µg range, effects of 3BC on fish reproduction can not be rouled out. Especially, as 3BC is lipophilic and bioaccumulated during 21 day of exposure with an average bioconcentration factor of 313±151. Furthermore the presence of other UV filters and hormonally active compounds, which may interact additively may adversely affect fish reproduction in contaminated aquatic systems. The pronounced effect of 3BC on reproduction, fecundity and fertility in fish after a 21 days exposure sheds new light on the potential hazard and risks of widely used UV filters.

Acknowledgment

We thank Christin Weisbrod, University of Zürich, for the excellent preparation and performance of histological sections, Beatrice Künzli, ETH Zürich, for water analysis, Helmut Segner and Stefanie Rossteuscher, Institute of Animal Pathology, University of Berne, for valuable comments on the gonad histology, Hans Geyer and Jocelyne Leclerc, Institute for Veterinary Anatomy, University of Zürich, for allowing to use the equipment for histology. Thanks are also due to Friedrich Jüttner and Jakob Pernthaler, Limnological Station, University of Zürich, and Hans-Rudolf Schmutz, Chemistry Department (FHNW), for their support. This work was supported by the Swiss National Science Foundation (NRP50, contract 4050-066554 to K. Fent).

References

Ankley, G. T., Jensen, K. M., Kahl, M. D., Korte, J. J., Makynen, E. A., 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*pimephales promelas*). Environ. Toxicol. Chem. 20(6), 1276-1290.

Ankley, G. T., Kahl, M. D., Jensen, K. M., Hornung, M. W., Korte, J. J., Makynen, E. A., Leino, R. L., 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (*Pimephales promelas*). Toxicol. Sci. 67, 121-130.

Ankley, G. T., Mihaich, E., Stahl, R., Tillitt, D., Colborn, T., McMaster, S., Miller, R., Bantle, J., Campbell, P., Denslow, N., Dickerson, R., Folmar, L., Fry, M., Giesy, J., Gray, L. E., Guiney, P., Hutchinson, T., Kennedy, S., Kramer, V., LeBlanc, G., Mayes, M., Nimrod, A., Patino, R., Peterson, R., Prudy, R., Ringer, R., Thomas, P., Touart, L., Van der Kraak, T., Zacharewski, T., 1998. Overview of a workshop

on screening methods for detecting potential (anit-)estrogenic/androgenic chemicals in wildlife. Environ. Toxicol. Chem. 17, 68-87.

Balmer, M., Buser, H. R., Müller, M. D., Poiger, T., 2005. Occurrence of some organic UV filters in wastewater, in surface waters, and in fish from Swiss lakes. Environ. Sci. Technol. 39, 953-962.

Buser, H. R., Balmer, M. E., Schmid, P., Kohler, M., 2006. Occurence of UV filters 4-methylbenzylidene camphor and octocrylene in fish from various swiss rivers with inputs from wastewater treatment plants. Environ. Sci. Technol. 40, 1427-1431.

Gimeno, S., Komen, H., Jobling, S., Sumpter, J., Bowmer, T., 1998. Demasculinisation of sexually mature male common carp, *Cyprinus carpio*, exposed to 4-tert-pentylphenol during spermatogenesis. Aquat. Toxicol. 43, 93-109.

Gronen, S. N., Denslow, N., Manning, S., Barnes, S., Barnes, D., Brouwer, M., 1999. Plasma vitellogenin level and reproductive impairment of male Japanese medaka (Oryzias latipes) exposed to 4-tert-octylphenol. Environ. Health Persp. 107, 385-390.

Hany, J., Nagel, R., 1995. Nachweis von UV-Filtersubstanzen in Muttermilch. Deut. Lebensm.-Rundsch. 91(11), 341-345.

Harries, J. E., Runnalls, T., Hill, E., Harris, C. A., Maddix, S., Sumpter, J. P., Tyler, C. R., 2000. Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). Environ. Sci. Technol. 34, 3003-3011.

Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J. P., Tylor, T., Zaman, N., 1997. Estrogenic activity in five United Kindom rivers detected by measurement of vitellogenesis in caged male trout. Environ. Toxicol. Chem. 16, 532-542.

Haubruge, E., Petit, F., Gage, M. J., 2000. Reduced sperm counts in guppies (Poecilia reticulata) following exposure to low levels of tributyltin and bisphenol A. Proc. Biol. Sci. 267(1459), 2333-2337.

Holbech, H., Norum, U., Korsgaard, B., Bjerregaard, P., 2002. The chemical UV-filter 3-benzylidene camphor causes an oestrogenic effect in an *in vivo* fish assay. Pharmacol. Toxicol. 91, 204-208.

Inui, M., Adachi, T., Takenaka, S., Inui, H., Nakazawa, M., Ueda, M., Watanabe, H., Mori, C., Iguchi, T., Miyatake, K., 2003. Effect of UV-screens and preservatives on vitellogenin and choriogenin production in male medaka (*Oryzias latipes*). Toxicology 194, 43-50.

Jensen, K. M., Korte, J. J., Kahl, M. D., Pasha, M. S., Ankley, G. T., 2001. Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*). Comp. Biochem. Physiol. C 128, 127-141.

Jobling, S., Coey, S., Withmore, J. G., Kime, D. E., Van Look, K. J., McAllister, B. G., Beresford, N., Henshaw, A. C., Brighty, G., Tyler, C. R., Sumpter, J., 2002. Wild intersex roach (Rutilus rutilus) have reduced fertility. Biol Reprod 67(2), 515-524.

Jobling, S., Sheahan, D., Osborne, J. A., Matthiessen, P., Sumpter, J. P., 1996. Inhibition of testicular growth in rainbow trout (Oncorhynchus mykiss) exposed to estrogenic alkylphenolic chemicals. Environ. Toxicol. Chem. 15, 194-202.

Junqueira, L. C., Carneiro, J., Long, J. A. (1986). The male reproductive system. Basic Histology. L. C. Junqueira. Los Altos, CA, Lange Medical Pub.**:** 468-484.

Kramer, V. J., Miles-Richardson, S., Pierens, S., Giesy, J. P., 1997. Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (*Pimephales promelas*) exposed to waterborne 17b-estradiol. Aquat. Toxicol. 40, 311-322.

Kunz, P. Y., Fent, K., in press. Activity of UV filters towards the estrogen and androgen receptor and comparison on *in vitro* and *in vivo* activity of ethyl 4-aminobenzoate in fish. Aquat. Toxicol.

Kunz, P. Y., Fent, K., submitted. Multiple hormonal activities of UV filters in vitro.

Kunz, P. Y., Galicia, H. F., Fent, K., 2006. Comparison of *in vitro* and *in vivo* estrogenic activity of UV filters in fish. Toxicol. Sci. 90, 349-361.

Länge, R., Hutchinson, T. H., Croudace, C. P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G. H., Sumpter, J. P., 2001. Effects of the synthetic estrogen 17a-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 20(6), 1216-1227.

Leino, R. L., Jensen, K. M., Ankley, G. T., 2005. Gonadal histology and characteristic histopathology associated with endocrine disruption in adult fathead minnow. Environ. Toxicol. Chem. 19, 85-98.

Miles-Richardson, S. R., Kramer, V. J., Fitzgerald, S. D., Render, J. A., Yamini, B., Barbee, S. J., Giesy, J. P., 1999a. Effects of waterborne exposure of 17b-estradiol on secundary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). Aquat. Toxicol. 47, 129-145.

Miles-Richardson, S. R., Pierens, S. L., Nichols, K. M., Kramer, V. J., Snyder, E. M., Snyder, S. A., Render, J. A., Fitzgerald, S. D., Giesy, J. P., 1999b. Effects of waterborne exposure to 4-nonylphenol and nonylphenol ethoxylate on secondary sex characteristics and gonads in fathead minnows (*Pimephales*

promelas). Environ. Res. Sec. A 80, S122-S137.
Mueller, S. O., Kling, M., Firzani, P. A., Mecky, A., Duranti, E., Shields-Botella, J., Delansorne, R., Borschard, T., Kramer, P. J., 2003. Activation of estrogen receptor a and ERb by 4-methylbenzylidene-camphor in human an rat cells: comparison with phyto- and xenoestrogens. Toxicol. Lett. 142, 89-101.
Nagtegaal, M., Ternes, T. A., Baumann, W., Nagel, R., 1997. UV-Filtersubstanzen in Wasser und Fischen. UWSF-Z. Umweltchem. Ökotoxikol. 9, 79-86.
Nash, J. P., Kime, D. E., Van der Ven, L. T. M., Wester, P. W., Brion, F., Maack, G., Stahlschmidt-Allner, P., Tyler, C. R., 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. Environ. Health Persp. 112(17), 1725-1733.
OECD (2001). Short-term reproduction test with the fathead minnow for identification of endocrine disrupting chemicals. Paris, OECD Environment Directorate.
Palace, V. P., Evans, R. E., Wautier, K., Baron, C., Vandenbyllardt, L., Vandersteen, W., Kidd, K., 2002. Induction of vitellogenin and histological effects in wild fathead minnows from a lake experimentally treated with the synthetic estrogen, ethynylestradiol. Water Qual. Res. J. Canada 37(3), 637-650.
Panter, G. H., Hutchinson, T. H., Länge, R., Lye, C. M., Sumpter, J. P., Zerulla, M., Tyler, C. R., 2002. Utility of a juvenile fathead minnow screening assay for detecting (anti-)estrogenic substances. Environ. Toxicol. Chem. 21(2), 319-326.
Parks, L. G., Cheek, A. O., Denslow, N. D., Heppel, S. A., McLachlan, J. A., LeBlanc, G. A., Sullivan, C. V., 1999. Fathead minnow (*Pimephales promelas*) vitellogenin: purification, characterisation and quantitative immunoassay for the detection of estrogenic compounds. Comparative Biochemistry and Physiology, Part C 123, 113-125.
Pawlowski, S., Sauer, A., Shears, J. A., Tyler, C. R., Braunbeck, T., 2004. Androgenic and estrogenic effects of the synthetic androgen 17a-methyltestosterone on sexual development and reproductive performance in the fathead minnow (*Pimephales promelas*) determined using the gonadal recrudescence assay. Aquat. Toxicol. 68, 277-291.
Poiger, T., Buser, H. R., Balmer, M., Bergqvist, P. A., Müller, M. D., 2004. Occurrence of UV filter compounds from sunscreens in surface waters: regional mass balance in two Swiss lakes. Chemosphere 55, 951-963.
Reolon, S., Ceccatelli, R., Faass, O., Stutz, E., Jarry, H., Wuttke, W., Lichtensteiger, W., 2004b. Endocrine acitivity and developmental toxicity of cosmetic UV filters - an update. Toxicology 205, 113-122.
Rodgers-Gray, T. P., Jobling, S., Kelly, C., Morris, S., Brighty, G., Waldock, M. J., Sumpter, J. P., Tyler, C. R., 2001. Exposure of juvenile roach (Rutilus rutilus) to treated sewage effluents induces dose-dependent and persistent disruption in gonadal duct development. Environ. Sci. Technol. 35, 462-470.
Routledge, E. J., Sumpter, J. P., 1997. Structural features of alkylphenolic chemicals associated with estrogenic activity. J. Biol. Chem. 272(6), 3280-3288.
Russell, L. D., Ettlin, R. A., Sinha Hikim, A. P., Clegg, E. D. (1990). Histological and histopathological evaluation of the testis. Clearwater, FL, Cache River Press.
Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B., Lichtensteiger, W., 2001. In vitro and in vivo estrogenicity of UV screens. Environ. Health Persp. 109, 239-244.
Schlumpf, M., Jarry, H., Wuttke, W., Ma, R., Lichtensteiger, W., 2004a. Estrogenic activity and estrogen receptor b binding of the UV filter 3-benzylidene camphor. Comparison with 4-methylbenzylidene camphor. Toxicology 199(2-3), 109-120.
Schlumpf, M., Schmid, P., Durrer, S., Conscience, M., Maerkel, K., Henseler, M., Gruetter, M., Herzog, I., Reolon, S., Ceccatelli, R., Faass, O., Stutz, E., Jarry, H., Wuttke, W., Lichtensteiger, W., 2004b. Endocrine acitivity and developmental toxicity of cosmetic UV filters - an update. Toxicology 205, 113-122.
Schreurs, R. H., Lanser, P., Seinen, W., Van der Burg, B., 2002. Estrogenic activity of UV filters determined by an in vitro reporter gene assay and in vivo transgenic zebrafish assay. Arch. Toxicol. 76, 257-261.
Schultz, T. W., Seward, J. R., Sinks, G. D., 2000. Estrogenicity of benzophenones evaluated with a recombinant yeast assay: Comparison of experimental and rules-based predicted activity. Environ. Toxicol. Chem. 19, 301-304.
Smith, R. J. F., 1974. Effects of 17a-methyltestosterone on the dorsal pad and tubercles of fathead minnows (*Pimephales promelas*). Can. J. Zool. 52, 1031-1038.
Smith, R. J. F., 1978. Seasonal changes in the histology of the gonads and dorsal skin of the fathead minnow, *Pimephales promelas*. Can. J. Zool. 56, 2103-2109.
Sperry, T. S., Thomas, P., 2000. Androgen binding profiles of two distinct nuclear androgen receptors in Atlantic croaker (*Micropogonias undulatus*). J. Steroid Biochem. Mol. Biol. 73, 93-103.
Sumpter, J. P., Johnson, A. C., 2005. Lessons from endocrine disruption and their application to other issues

concerning trace organics in the aquatic environment. Environ. Sci. Technol. 39(12), 4321-4332.
Sullivan, C. V., 1999. Fathead minnow (*Pimephales promelas*) vitellogenin: purification, characterisation and quantitative immunoassay for the detection of estrogenic compounds. Comparative Biochemistry and Physiology, Part C 123, 113-125.
Tilton, F., Benson, W. H., Schlenk, D., 2002. Evaluation of estrogenic activity from a municipal wastewater treatment plant with predominantly domestic input. Aquat. Toxicol. 61(3-4), 211-224.
Trudeau, V. L., Wade, M. G., Van Der Kraak, G., Peter, R. E., 1993. Effects of 17b-estradiol on pituitary and testicular function in male goldfish. Can. J. Zool. 71(1131-1135).
Tyler, C. R., Jobling, S., Sumpter, J. P., 1998. Endocrine disruption in wildlife: a critical review of the evidence. Crit. Rev. Toxicol. 28(4), 319-61.
Tyler, C. R., Van Aerle, R., Hutchinson, T. H., Maddix, S., Trip, H., 1999. An in vivo testing system for endocrine disrupting in fish early life stages using induction of vitellogenin. Environ. Toxicol. Chem. 18(2), 337-347.
Wells, K., Van Der Kraak, G. (1998). The ability of mammalian antiandrogens to bind to androgen receptors in the brain of rainbow trout (*Oncorhynchus mykiss*). Proceedings of SETAC 19th Annual Meeting, Charlotte, NC, USA, SETAC.

Chapter 7

Assessment of hormonal activity of UV filters in tadpoles of frog *Xenopus laevis* at environmental relevant concentrations

Petra Y. Kunz, Héctor F. Galicia and Karl Fent

published in *Marine Environmental Research, 2004, 58, 431-435*

Abstract

Residues of UV filters used as ultraviolet absorbers in sunscreens, cosmetics and in light protection have been found in surface water and fish. Recently some of them were shown bearing estrogenic activity, however, very little is known about possible adverse effects to aquatic life. Here we investigate whether two UV filters, 4-methylbenzylidene camphor (4-MBC) and 3-benzylidene camphor (3-BC), interfere with the thyroid and sex hormone system during frog metamorphosis. *Xenopus laevis* tadpoles were exposed to 1, 5 and 50 µg/L of 4-MBC and 3-BC, respectively, for 35 days (stage 52-66). The rate of metamorphosis was not affected, and no obvious differences in body and tail length compared to controls were observed. Neither 4-MBC, nor 3-BC lead to effects on the sex ratio or gross gonad morphology of *X. laevis* at stage 66. Our results indicate that these UV filters do not negatively affect the thyroid system and sex ratio of frogs at environmental concentrations.

Introduction

UV filters used in sunscreens, cosmetics and light protection of materials may contaminate the aquatic environment in relevant concentrations, since these compounds are used in rather high volumes, and display critical properties such as high lipophilicity. Recently, residues of UV filters have been found in water (Poiger, Buser & Müller, 2001) and fish (Nagtegaal, Ternes, Baumann & Nagel, 1997) ranging from 2-125 ng/L and 26-3'100 µg/kg , respectively. Some of them displayed estrogenic activity *in vitro* (Miller et al., 2001; Schreurs et al., 2002) and *in vivo* in rats (Schlumpf et al., 2001) and fish (Holbech et al., 2002). Still bioaccumulation of UV filters and possible sex hormone disrupting effects to aquatic life are largely unknown, along with the uncertainty whether other hormone systems like the thyroid system are also concerned. This hampers the environmental hazard and risk assessment of these chemicals.

This study is aimed at elucidating possible activities of the two UV filters, 4-methylbenzylidene camphor (4-MBC) and 3-benzylidene camphor (3-BC), towards the thyroid and sex hormone system of frogs *Xenopus laevis,* especially as some endocrine disrupting chemicals have been demonstrated to interfere with both, the thyroid and sex hormone system of amphibians (Goleman, Carr & Anderson, 2002; Moriyama et al., 2002).

Material and Methods

Tadpoles of *X. laevis* were held in well-aerated artificial tab water (Kloas, Lutz & Einspanier, 1999) and a 12 h/12 h light/dark cycle at 22±1°C. For exposure using a 48 h (weekdays) and 72 h (weekends) aqueous static-renewal procedure, 30 randomly selected tadpoles each were placed in 2 replicate stainless steel tanks (10 L) and exposed to 4-MBC and 3-BC, respectively, for 35 days from stage 52 to 66). A control, solvent control and positive control for thyroid activity (1 µg/L thyroxin, T4) were included. Nominal concentrations of both 4-MBC and 3-BC were 1, 5 and 50 µg/L. Actual concentrations of repeatedly taken water aliquots were determined after solid phase extraction and GC/MS analysis (Poiger, Buser & Müller, 2001).

Developmental stage, whole body and tail length of the tadpoles were determined in our experiments for possible effects of UV filters on metamorphic development, growth rate and tail resorption. All measurements were performed on day 0, 7, 14, 21, 28 and 35 of exposure. At the end of the experiment possible effects of the UV screens on the sex hormone system were investigated by visual inspection of gonads, thereby assessing sex ratio and gross gonad morphology.

Statistical comparisons were performed using Kruskal-Wallis one-way ANOVA (on ranks, Dunn's test) for development stages, body and tail length on day 0 to 28 and the Chi-square test for sex ratio.

Results

Actual concentrations were close to nominal values at 0 h for all exposure groups, ranging between 93-115% and 84-100% for 4-MBC and 3-BC, respectively. Actual concentrations of the of 1 µg/L group decreased during the 72 h exposure to 39 % and 87% for 4-MBC and 3-BC, respectively, and it is assumed that this also holds for the 5 and 50 µg/L exposure groups. Mortality was less than 3% in all treatment groups and no apparent behavioural differences were observed between treatments.

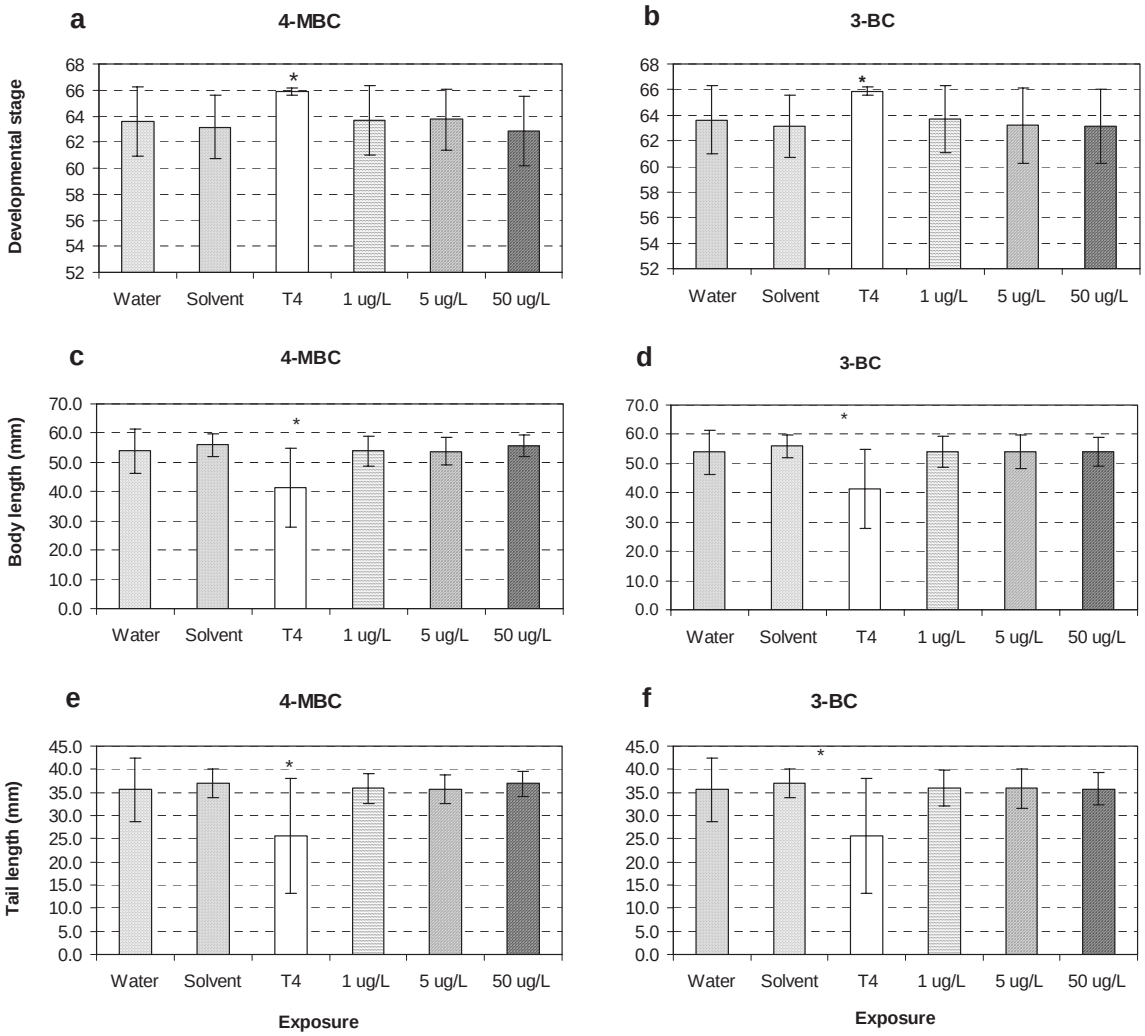


Figure 1 Effects on developmental stages of X. laevis tadpoles after 35 days of exposure with 4-methylbenzylidene camphor (a) and 3-benzylidene camphor (b) on day 28. Effects on the body length of tadpoles after exposure with 4-methylbenzylidene camphor (c) and 3-benzylidene camphor (d) on day 21. Effects on tail length of tadpoles after exposure with 4-methylbenzylidene camphor (e) and 3-benzylidene camphor (f) on day 21. Values are shown as mean ± SD (standard deviation). * denotes significant difference from controls ($p<0.001$).

Neither 4-MBC nor 3-BC affected the rate of metamorphosis. Recorded developmental stages, body and tail lengths of *X. laevis* tadpoles exposed to 1, 5 and 50 µg/L of 4-MBC and 3-BC, respectively, did not differ from the tadpoles of the water and solvent controls (Figs. 1a, 1b). At all concentrations growth, tail development and tail resorption were not affected by the UV filters (Figs. 1c-1f). In contrast, the positive control (1 µg/L T4) accelerated metamorphosis and differed significantly ($p<0.001$) from all other treatment groups in the developmental stage, as well as body and tail length. The exposure of tadpoles to 1, 5 and 50 µg/L of 4-MBC and 3-BC, respectively, had no significant effect on sex ratio, nor gross gonad morphology when compared with controls (Figs. 2a, 2b).

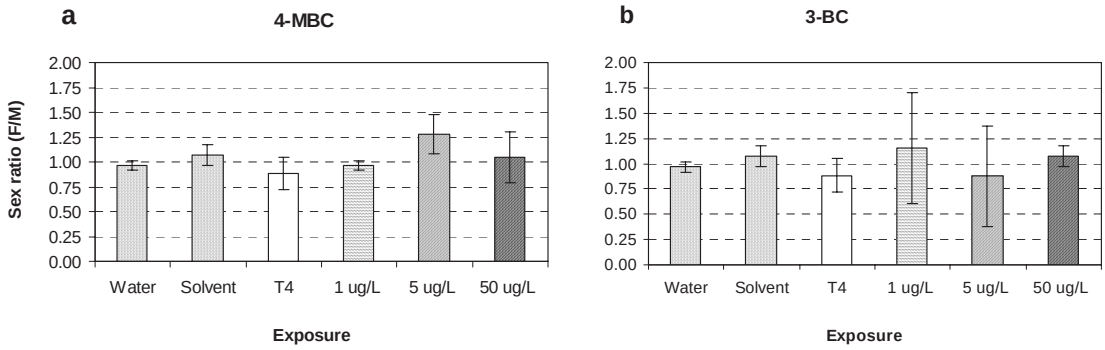


Figure 2 Effects of 4-methylbenzylidene camphor (a) and 3-benzylidene camphor (b) on the sex ratio (number of females/number of males)(n= 2 x 30 per treatment group) of X. laevis after 35 days of exposure. Values are shown as mean ± SD (standard deviation). * denotes significant difference from controls ($p<0.001$).

Discussion

The goal of this study was to elucidate whether the two UV filters 4-MBC and 3-BC adversely affect the thyroid and sex hormone system of frogs *Xenopus laevis.* Recently some endocrine disrupting chemicals have been demonstrated to interfere with both, the thyroid and sex hormone system of amphibians (Goleman, Carr & Anderson, 2002; Moriyama et al., 2002). Tail resorption and developmental stage are important parameters for the assessment of thyroidogenic activity (DeVito et al., 1999). Anti-thyroid and thyroid-mimicking substances can be detected through delayed or enhanced metamorphosis resulting in lower or higher developmental stages, respectively (Fioramonti et al., 1997; Goleman et al., 2002). Therefore developmental stage, whole body and tail length of the tadpoles were determined in our experiments for possible effects of UV filters on metamorphic development, growth rate and tail resorption.

Neither of the tested UV filters did affect the rate of metamorphosis and thus, they do not exhibit thyroid-mimicking or anti-thyroid activity. Recorded developmental stages, body and tail lengths of *X. laevis* tadpoles exposed to 1, 5 and 50 µg/L of 4-MBC and 3-BC, respectively, did not differ from the tadpoles of the water and solvent controls, indicating that neither growth,

nor tail development and tail resorption were affected by the UV filters at these concentrations. In contrast, the positive control (T4) accelerated metamorphosis and differed significantly from all other treatment. The exposure of tadpoles to 1, 5 and 50 µg/L of 4-MBC and 3-BC, respectively, had no significant effect on sex ratio, nor gross gonad morphology when compared with controls.

Our results show that the exposure of *X. laevis* tadpoles to the two UV filters at environmentally relevant concentrations did neither affect metamorphosis, nor sex ratio. Whereas tadpoles of the positive control (T4) showed smaller body and tail lengths and reached metamorphosis earlier, thus confirming the reliability of this assay at least for compounds with strong thyroid activity. Our preliminarily results showing no impact of 4-MBC and 3-BC on sex ratio and gonad morphology do not rule out other effects on the sex hormone system. Gonadal development in *X. laevis* takes place prior to stage 56, at which the differentiation in female and male gonads can be determined. At stage 51-53 an undifferentiated gonad is established and after exposure to estradiol at stages 51–54 around 50 % of the tadpole gonads showed normal ovaries, whereas the other 50 % had abnormal gonads, corresponding to ovotestes, in which normal development of genetic males was disrupted (Villalpando & Merchant-Larios, 1990). Based on these findings that partial effects of estradiol treatment occurred at stages 51-54, it cannot be ruled out, that in our studies, lasting from stage 52-66, ovotestes or vitellogenin may have been induced, despite the lack of effects on the sex ratio.

4-MBC and 3-BC showing weak estrogenicity (Holbech et al., 2002; Schlumpf et al., 2001) may only display effects on the thyroid and sex hormone system at much higher concentrations or at prolonged exposure of the tadpoles. The former is supported by the fact that estrogenic activity was lacking after short-term exposure of fish to 254 µg/L 4-MBC, but occurred at higher concentrations *in vitro* (Schreurs et al., 2002). Differences in exposure route, bioavailability and the capability of UV filters for binding to the different hormone receptors in different species may account for the differences in the studies. Ongoing studies in our laboratory will further test the hypothesis whether the UV filters possess hormonal activity.

Acknowledgements

This work was supported by the Swiss National Science Foundation (NFP 50, contract 4050-66554 to K. Fent). We thank Prof. D. Rungger, Institute of Zoology, University of Geneva, for generously providing *X. laevis* tadpoles, Dr. C. Schanné and Dr. T. Gries, Springborn Smithers Laboratories (Europe) AG, R. Opitz, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin and Dr. H. Schmutz, FHBB, for advice and Prof. F. Jüttner, University of Zürich, for reading the manuscript.

References

DeVito, M., Biegel, L., Brouwer, A. & Brown, S. (1999). *Environmental Health Perspectives, 107,* 407-415.

Fioramonti, E., Semlitsch, R.D., Reyer, H.U. & Fent, K. (1997). *Environmental Toxicology and Chemistry, 16,* 1940-1947.

Goleman, W.L., Carr, J.A. & Anderson, T.A. (2002). *Environmental Toxicology and Chemistry, 21,* 590-597.

Holbech, H., Norum, U., Korsgaard, B. & Bjerregaard, P. (2002). *Pharmacology and Toxicology, 91,* 204-208.

Kloas, W., Lutz, I. & Einspanier, R. (1999). *Science of the Total Environment, 225,* 59-68.

Miller, D., Wheals, B.B., Beresford, N. & Sumpter, J.P. (2001). *Environmental Health Perspectives, 109,* 133-138.

Moriyama, K., Tagami, T., Akamizu, T., Usui, T., Saijo, M., Kanamoto, N. *et al.* (2002). *Journal of Clinical Endocrinology & Metabolism, 87,* 5185-5190.

Nagtegaal, M., Ternes, T.A., Baumann, W. & Nagel, R. (1997). *UWSF-Z Umweltchemie und Ökotoxikologie, 9,* 79-86.

Poiger, T., Buser, H.R. & Müller, M.D. (2001). Verbrauch, Vorkommen in Oberflächengewässern und Verhalten in der Umwelt von Substanzen, die als UV-filter in Sonnenschutzmitteln eingesetzt werden. Wädenswil. Eidgenössische Forschungsanstalt für Obst-, Wein- und Gartenbau, CH-8820 Wädenswil. 1-45.

Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B. & Lichtensteiger, W. (2001). *Environmental Health Perspectives, 109,* 239-244.

Schreurs, R., Lanser, P., Seinen, W. & Van der Burg, B. (2002). *Archives of Toxicology, 76,* 257-261.

Villalpando, I. & Merchant-Larios, H. (1990). *International Journal of Developmental Biology, 34,* 281-285.

Chapter 8

General Conclusions and Outlook

This dissertation focused on the hormonal activity of UV filters towards the aquatic organisms. Recently, organic UV filters gained environmental relevance, because of their demonstrated estrogenicity, antiestrogenic and antiandrogenic activity and their detection in lake and wastewater, but also in fish. However, due to the lack of data on the ecotoxicological and hormonal activity of UV filters, it remains unclear, whether environmental concentrations of UV filters negatively affect the hormone system of aquatic organisms. Currently an ecotoxicological risk assessment for aquatic organisms is premature, because the data base is not sufficient.
This dissertation was aimed at filling some of these significant gaps by the use of a series of ecotoxicological *in vitro* and *in vivo* assays. The overall goal was to evaluate the estrogenic, antiestrogenic, androgenic and antiandrogenic activity *in vitro* of a series of single UV filters and estrogenic mixtures activities thereof. In addition the estrogenic and thyroid activity *in vivo* was evaluated in order to understand the hormonal activity and ecokinetics in important aquatic organisms.

Hormonal activity of UV filters in vitro

Hormonal activity of single UV filters. Our findings demonstrate that many UV filters occurring in aquatic systems possess multiple hormonal activities *in vitro*, including estrogenicity, antiestrogenicity, androgenicity and antiandrogenicity through interactions with hERα and/or hAR. For most of the UV filters with multiple hormonal activities residues in the aquatic environment and in biota are not yet known, and therefore their environmental relevance remains elusive. The fact that all 18 UV filters and one metabolite showed hormonal activities - surprisingly most of them multiple activities - reveals a complex picture of their activities. Antiestrogenicity and antiandrogenicity *in vitro* seems even more important than estrogenicity. Moreover, androgenic activity of six UV filters is demonstrated for the first time.

Our results support the fact that many estrogenic compounds may have more than one mode of action, and that their diverse hormonal activities are more complex than previously thought. The antiestrogenic, antiandrogenic and androgenic activities of UV filters *in vitro* are of significant scientific and practical interest. Despite the limitations of yeast systems such as lower metabolic capacity as compared to many animal cell systems, they are an elegant and efficient tool to identify endocrine disrupting compounds that may further be investigated *in vivo*, especially regarding adverse effects in fish.

Hormonal activity of UV filter mixtures. Recent studies on hormonal activities of UV filters suggest that these compounds possess only endocrine disrupting properties at rather high, environmentally not relevant concentrations. Although we found in the yeast hERα assay

that most of the UV filters exert multiple hormonal activities at concentrations that are orders of magnitude higher than in the environment, wide distribution of and exposure to UV filter mixtures may have environmental consequences due to additive effects. Thus the UV filters 4MBC, BP3, BP4, OMC, OC and HMS that were repeatedly detected in the aquatic environment, may contribute with their multiple hormonal activities in a complex manner to the mixture of endocrine disrupting chemicals already present in surface water and fish. Our *in vitro* findings on estrogenic mixture activities of pure and parital hERα agonistic UV filters seem to support this hypothesis. Especially the pronounced synergistic effects of multi-component mixtures of UV filters below the NOEC indicate that low UV filter concentrations present in the environment may produce relevant estrogenic activity on their own or lead to enhanced estrogenic activities of other more potent xenoestrogens or E2, depending on the mixture components.

Indeed, concentrations of single UV filters in the NOEC mixtures were mostly in the µg/L range when eliciting highest estrogenic activities *in vitro*. These are effect concentrations that are close to residual concentrations of UV filters found in the environment. Our findings on UV filter mixtures furthermore suggest that partial agonistic UV filters not necessarily lead to a reduced overall mixture activity and antagonism. In order to find out if our *in vitro* findings for mixtures will translate into animal cell-lines and to effects *in vivo* in fish, further studies are needed to support our results.

Hormonal activity of UV filters in vivo

Estrogenic activity of UV filters in fish. In order to investigate whether our *in vitro* results on estrogenicity translate to *in vivo* activity we exposed juvenile fathead minnows for 14 days to 9 different UV filters. Seven UV filters were found to be estrogenic *in vitro* (BP1, BP2, BP3, BP4, DHB, Et-PABA and 3BC), whereas the others possessed no estrogenicity (4MBC, OMC). Of these UV filters, 3BC, BP1 and BP2 showed estrogenic activity in fathead minnows. 3BC was the most estrogenic UV filter in juvenile fathead minnows and led to dose dependent induction of VTG at 435 and 953 µg/L. The estrogenicity detected for BP1 at 4919 µg/L and BP2 at 8783 µg/L was considerably weaker. The other UV filters (4MBC, OMC, BP3, BP4, 4DHB) did not induce VTG up to the highest concentrations.

In the environment only a few UV-filters such as OC, 4MBC, BP3 and BM-DBM have been analysed so far and 3BC, BP1 and BP2 which were found in our study to be estrogenic are not among them. However, concentrations of UV filters were detected up to 125 ng/L in lake water, up to 2.7 µg/L in treated wastewater, and up to 3.1 µg/g in fish, and thus considerably lower than the UV filter concentrations to induce VTG. Our data on VTG induction suggest that one

single UV filter may probably not pose a hazard to fish. However, different UV filters may act additively (chapter 3) (Heneweer *et al.* 2005) as indicated for other endocrine disrupters (Routledge *et al.* 1998). Moreover, long-term exposure to UV filters may affect fish reproduction at much lower concentrations.

Predictive value for in vivo activity of the yeast hER and rtER in vitro assays. Considering the vast number of compounds to be tested for possible endocrine activity, it is important to employ appropriate *in vitro* systems. Thus in order to evaluate if the used *in vitro* assays, carrying either the hERa or rtERa, have any predictive value to estimate *in vivo* activity in fish, we compared our *in vitro* and *in vivo* findings for the 9 UV filters investigated in juvenile fathead minnows. The advantage of *in vitro* assays is that they are cost effective and allow for rapid screening of a large number of compounds, but they have limitations, which may result in unreliable predictions.

We found that all compounds, except OS, were equally estrogenic *in vitro* in both assays despite the lower activity of E2 in the rtERα assay. When comparing *in vitro* activities of the two systems with fish *in vivo* activity, we found that the rtERα *in vitro* data were more accurate for estimating *in vivo* activity. Hence estrogenic activity of chemicals is best assessed by the use of a tiered approach with a combination of *in vitro* and *in vivo* assays of the same phyla, and if possible, of the same species. In this tiered approach, the predictive power of *in vitro* systems is enhanced and cost intensive *in vivo* studies can be reduced by employing species-specific *in vitro* assays. Hence, environmental risk assessment should be based on combined, complementing and appropriate species-related *in vitro* and *in vivo* assays for hormonal activity.

Effects of 3BC on fish reproduction. We investigated the most potent UV filter detected in the short-term experiment with juvenile fathead minnows, 3BC, for possible adverse effects on reproduction. 3BC significantly affected the reproduction of this fish. Females stopped reproducing immediately after the onset of exposure at a concentration of 74 µg/L 3BC and higher. To our surprise, the lowest observed effect concentration for the most sensitive parameter, gonadal histology, was 3 µg/L. This parameter is an order of magnitude more sensitive than VTG induction. Hence the question arises whether the hormonal activity of a compound is underestimated by determining VTG induction alone, and that also BP1 and BP2, that were found active in fish, nevertheless pose a potential risk towards fish (chapter 4).

Environmental concentrations of 3BC in water and fish have not yet been reported, but may reach the same level than other UV filters such as 4MBC. If they are in the low n/L to µg/L range as other UV filters, effects of 3BC on fish reproduction can not be ruled out, especially as 3BC is lipophilic and bioaccumulated during the 21 days of exposure with a bioconcentration factor of 207 to 648. Furthermore the presence of other UV filters and hormonally active compounds,

which may interact additively may adversely affect fish reproduction in contaminated aquatic systems. The pronounced effect of 3BC on reproduction, fecundity and fertility in fish after a 21 days exposure has significant consequences for potential hazard and risks of widely used UV filters.

Effects of 3BC and 4MBC on frog metamorphosis. As another important representative of aquatic organisms, tadpoles of frogs *Xenopus laevis* were also studied for their susceptibility towards two UV filters. Because metamorphosis is a critical period for endocrine disruption, we investigated, whether 4MBC and 3BC interfere with the thyroid and sex hormone system during frog metamorphosis. Neither 4MBC nor 3BC did affect the rate of metamorphosis, and no obvious differences in body length and tail length compared to controls were observed. Also the UV filters did not lead to alterations of the sex ratio of *X. laevis* tadpoles. Our findings indicate that neither 4MBC nor 3BC negatively affect the thyroid system and sex development of frogs at concentrations expected to be found in the environment.

A tentative hazard assessment for UV filters

There are still more data needed such as the bioaccumulation potential and mixture activity of UV filter in fish, before an accurate ecotoxicological risk assessment for aquatic organisms can be performed. However, in order to evaluate whether a further risk assessment has to be performed, we calculated PEC/PNEC values based on our results and data on environmental concentrations from the literature.

Environmental concentrations of 3BC, BP1, BP2 and Et-PABA are not existent, therefore we assume similar environmental concentrations of structurally similar compounds such as 4MBC, BP3 and PABA as an approximation for the predicted exposure concentration (PEC). When lipid based weights in fish were used instead of concentrations in water, we divided the concentrations found in fish by a factor of 20, based on the assumption that the lipids content in fish are approximately 5%.

The predicted no effect concentration (PNEC) was calculated as follows:

PNEC = lowest exposure concentration / safety factor

For estimating the PNEC, we used the median value of the most significant reproduction parameter, which was the alteration of the gonadal histology for 3BC and VTG induction for BP1, BP2 and Et-PABA. We used a safety factor of 100, which is used when acute LC 50 values for fish, crustaceans or algae are known, or when chronic values for one of this group

exist. Values for acute and chronic toxicity parameters and environmental concentrations and calculations for our tentative hazard assessment are found in Table 1:

	Toxicity acute (mg/L)	chronic (mg/L)	LOEC (µg/L)	Safety factor	PNEC (µg/L)	PEC (µg/L)	PEC/ PNEC	Risk
3BC	0.141[1)]	>1	3	100	0.03	0.1 [4)]	3.3	YES
BP1	3.882[2)]	>5	4'919	100	49.2	0.5 [5)]	0.010	NO
BP2	3.882[2)]	>10	8'783	100	87.8	0.5 [5)]	0.005	NO
Et-PABA	62.00[3)]	>10	4'393	100	43.9	0.5 [5)]	0.011	NO

[1)] 3BC, LC50 in fish 96 h, rainbow trout

[2)] BP3, LC50 in fish 96 h, rainbow trout

[3)] Et-PABA, LC50 in fish 24 h, rainbow trout, Invest Fish Control Rep. No. 87, Fish Wildl. Serv., Bur. Sport Fish. Wildl., U.S.D.I., Washington, D.C.; 50, 1979

[4)]Balmer et al., 2005: mean concentration of 4MBC in wastewater effluent.

[5)]Lorraine and Pettigrove, 2006: mean concentration of benzophenone in raw drinking water.

The tentative risk assessment indicates that a hazard and risk of 3BC exists for fish, but not for BP1, BP2 and Et-PABA. However, the LOEC concentrations we used for BP1, BP2, and Et-PABA are based on VTG induction, which may be two orders of magnitude less sensitive than histology towards endocrine disruption, which we clearly demonstrated for 3BC. Therefore, a potential risk of these UV filters cannot be ruled out.

Final remarks and outlook

Our study reveals a novel and more detailed picture of the hormonal activity of UV filters, as single compounds and in mixtures, *in vitro* and *in vivo.* It discloses unexpected multiple hormonal activities and synergistic properties of these compounds *in vitro* and an unexpected negative effect on fish reproduction by the UV filter 3BC at nearly environmentally relevant concentrations *in vivo*. Hence, diverse hormonal activities of UV filters found in this study are of significant scientific and practical interest.

Generally we think that in order to perform an adequate risk assessment for UV filters it seems unavoidable to consider several levels of investigation. First, screening of UV filters for multiple hormonal activities and mixture activities *in vitro* reveals UV filters that may also have activity *in vivo.* Second, reproduction experiments in fish provide more sensitive and informative data on the endocrine disrupting properties of an UV filter and third, compound mixtures have to

be taken into account when investigating endocrine disrupting properties of UV filters towards aquatic organisms.

As it is not known to what extend the estrogenic UV filters occur in the environment and in fish, a comprehensive hazard and risk assessment is premature. Forthcoming studies should determine environmental concentrations of UV filters with hormonal activity and should relate them to effect concentrations. For hazard and risk assessment, potential effects on reproduction, fecundity and fertility in fish are necessary, as well as bioaccumulation studies. Moreover, UV filters may have multiple hormonal activities such as antiestrogenicity, androgenicity and antiandrogenicity besides estrogenicity and their activity in mixtures has to be considered in addition. Whether these multiple hormonal activities and mixture activities are reflected *in vivo* in fish and whether reproduction effects occur will be investigated in our laboratory in forthcoming studies.

Curriculum Vitae

Name	Kunz
First Name	Petra Yvonne
Date of Birth	2nd of October 1971
Place of Birth	Schaffhausen
Hometown	Maur/ZH
Nationality	Swiss

Education

1995	"**Matura Typus E**", Ostschweizer Maturitätsschule für Erwachsene, Frauenfeld, Switzerland
1995-2001	**Studies of Biology at the University of Basel (Biology I)** (Population Biology, Vertebrate Biology, Parasitology, Plant Ecology, Conservation Biology)
2000-2001	**Master Thesis** in population biology: "Activity Patterns and home range of the small frugivorous bat, *Artibeus watsoni* and its implications on seed dispersal" at the University of Ulm, Department of Experimental Ecology in the group of Prof. Elisabeth Kalko and at the Smithsonian Tropical Research Institute (STI), Barro Colorado Island, Panama.
2002-2006	**Ph.D. study** at the University of Zürich: "Hormonal activity of UV filters in the aquatic ecosystem" in the group of PD Dr. Karl Fent, University of Applied Sciences Northwestern Switzerland, School of Life Sciences, Muttenz, Switzerland